The FDA Banned Fruit seeds & Cannabis hemp To crush the competition & Profit Of The Deaths☠Of Millions

The FDA Ties Between The Rockefeller Family Chemistry And The Pharmaceutical Cancer Industry
The F.D.A In this video /read me by Anonymous Hacking Group] we will expose the ties between the Rockefeller family chemistry & the pharmaceutical cancer industry.
The Food & Drug administration began life as the Division of the Bureau of Chemistry, long before changing its name to the F.D.A. Food & Drug Administration.
It is believed that its name was changed to conceal its chemical industry agenda to blur the dividing line between what is safe.
It is always provided a type of legal immunity for companies that put chemicals into foods, and to pharmaceutical companies via so called its approvals"; which leave citizens without legal recourse against the chemical industry.
these are the original chemists from the Bureau of Chemistry, in 1909 -- that later become the F.D.A.. Bureau of the Food & Drug Administration In 1913. Its true job was, and is, to "approve" and sanction products from the chemical industry, and thereby legally shield the chemical industry from us by declaring what is "safe.
They routinely privately gather data to share with their chemical industry partners to mitigate liability.
For example, contamination information that they hide from the public. These are "public relations excuse issues" for them.
Good luck winning a case in a United States Court against a billion dollar chemical company whose products are "F.D.A. approved" and given a G.R.A.S. (Generally' Recognized As Safe) SAFE HARBOR STATUS.
These shield the chemical companies, and give them plausible deniability.
The modern F.D.A. came into being in 1913 -- the same year that the Rockefeller Foundation was created.
The F.D.A. works hand-in-hand with the Rockefeller Foundation & the American Medical Association.
The Rockefeller Foundation was founded in 1913, with its main emphasis upon medicine & medical education.
The initial Rockefeller medical school donations totaled over $550,000,000. in 1913 alone.
The Rockefeller Foundation gave money to 18 medical schools across 14 countries.
It's partners at the F.D.A. began an aggressive campaign of suppressing medicines that competed with the chemical industry.
Dr Richard J. Crout, Director of the F.D.A. Bureau of Drugs said in Spotlight on January 18, 1982, I never have & never will approve a new drug to an individual, but only to a large pharmaceutical company with unlimited finances."
Suppressed were the natural medicines that their industry could not legally control & monopolize with "drug" (chemical) patents.
Natural substances can never be patented.
This is why every natural and non-toxic therapy is automatically called "quackery" by the F.D.A., the chemical industry & the medical establishment. Only unnatural synthetic (ie, non-organic toxic), from CRUDE OIL/TAR petrochemicals may be patented, & thus, non-toxic medicines are a threat to the corupped business of poison under the name of medicine.

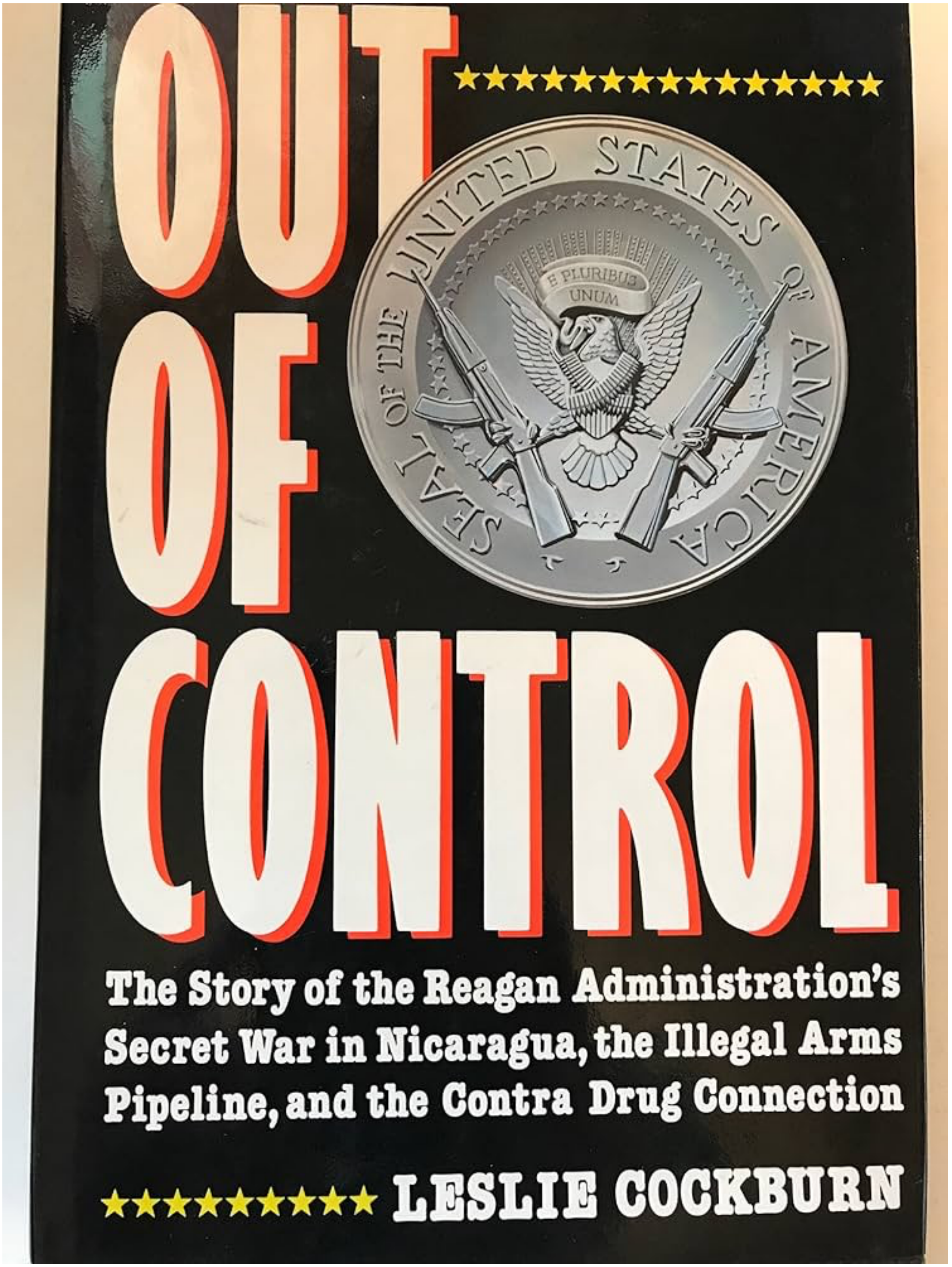
A report from the McGill University Press Said in 1919, a five million dollar Rockefeller Foundation gift to certain Canadian medical schools helped bring Canadian medical education to the 20th century.
Medicine was not the lucrative market that it is today, so medical schools were begging for the Rockefeller grants that were being handed out to COMPLIANT SCHOOLS.
COMPLIANCE to the Rockefeller stipulations required abandoning traditional & natural medicines in lieu of a new genocide generation of BIO petrochemicals for drugs, & trashing ENTIRE LIBRARIES of past procedures.
What happened was a preamble to book burnings, elsewhere, and in some cases, the F.D.A. did indeed have book burnings.
Those were dangerous times for traditional naturopathic (non-chemical industry) doctors.
All dissenters who embraced the old time-tested holistic & naturopathic methodologies of healing would henceforth be demonized as "quacks" in formal medical training.
That was despite some of those "quacks" having Nobel Prizes in medicine, & vastly superior results.

It was a carrot & stick strategy. compliant schools were given the carrot (Rockefeller money).
Competing doctors were given the stick (F.D.A. police actions).
Some Doctors ended up dead or sentenced to life in prison for having cured to many patients with "unapproved" medicine.
It is still happening.
Don't you feel like being raped by Rockefeller's F.D.A. by reading this?
At stores selling organic (non toxic-chemical) foods.
certain cancer-killing vitamins or even raw milk. it is not just the doctors with cures anymore.
Curing is illegal, by the way, since no cures are ever approved".
Cures mean freedom from chemical dependence: freedom from City Hall, Rockefeller, F.D.A. 201(ff)(3)(B)(ii), DuPont, D.E.A. 6630507, A.C.S, the N.I.C., the A.M.A. & the corrupt major oncology centers.
An unholy alliance formed between the American Medical Association, the F.D.A., & the Rockefeller Foundation to insure compliance from the Medical schools.
The Rockefeller Foundation Frequently insisted that medical schools place Rockefeller employees on their board of directors.
The general Education Board of the Rockefeller Foundation, also approached in 1914, pledged $5000,000 on the condition that the University raised $1.5,000,000 by January 1, 1916 & paid clinical faculty [allopathic doctors only] on a full-time basis.
With the Bribed money and the hope of GEB funding, the Yale Corporation & Hospital signed the affiliation contract with the Hospital before the deadline of July 1, 1914." --
Yale University Medical School Web Site.
The new legally enforceable medical monopoly paralleled the past Rockefeller monopoly from the petro, bio CRUDE OIL/TAR chemical

industry of times past. Instead of owning all petroleum, the Rockefeller SATANIC Empire now controlled virtually all POISONOUS medicine whereby the Rockefeller can now sell many petroleum POISONOUS chemicals, (POISONS in fact) that cannot be utilize as fuel, plastics or synthetics.
He owned the lion's share of the chemical industry, which would later be called the "pharmaceutical" industry.
Throughout his life, John D. Rockefeller, Sr. refused to take his own medicine, for it is POISON.
He used traditional holistic medicines for his own health his entire life. Alongside many of his friends at the F.D.A. & the A.M.A..
After the hijack of the medical schools, the true carnage of polio, heart disease & cancer exploded.
"I had a brain cancer specialist sit in my living room & tell me that he would never take radiation if he had a brain tumor, & I asked him, but, do you send people for radiation?' & he said; of course, I'd be drummed out of the hospital if I didn't,''' Said Dr Ralph Moss, Ph.D. winner of 1937 Nobel prize of medicine
Gyorgyi, Ph.D., discoverer of vitamin C, winner of 1937 Nobel Prize for medicine said "The American Cancer Society tried to ruin my research foundation. Guess when this organization [American Cancer Society) was founded & by whom.
It was founded by John D. Rockefeller Jr. in that MAGIC year 1913, while "donating" at Harvard University.
Do you believe that they will someday announce a cure, & cut themselves off from the truckloads of money?
A better question would be why vitamin C. was a threat to their mission.
What is their maine aim again, oh yah; DEPOPULATION while Gaining the whole World for themselves.

According to the U.S. Census, cancer is the 2nd largest killer in the United States, but most of those people die from the POISONS, ah I meen medicine's; not the cancers.
Pharmaceutical drugs are the fourth largest killer.
The U.S. has the most dangerous drugs, & yet the most expensive medical system in the World, it causes 60% of all U.S. bankruptcies.
Dr. Robert Atkins, M.D., (creator of "the Atkins Diet") said "There is not one, but many Cures for cancer available, but they are all being systematically suppressed by the A.C.S, the N.I.C. & the major oncology centers.
They have too much of an interest in the status quo."
Cancer cure Vitamin B17. (laetrile. In 2007 bitter almonds (not regular almonds) were becoming popular with alternative medicine due to their vitamin B17. content.
Health food stores stopped selling B17. in the form of apricot seeds in 2002, due to armed F.D.A raids.
There were reports everywhere of cancers being killed merely from patients eating heaping servings of bitter almonds daily.

Soon there would be almond extract capsules, so that these same patients would need only to take one cheap, non-toxic pill per day.
It did not bode well for the pharmaceutical industry. Hundreds of billions of dollars were about to be lost.
The F.D.A. openly banned apricot seeds, & the vitamin B17. extract, because it was successfully curing cancers as an "unapproved drug".
The British Government soon followed suit, & did the vary same thing during 2002.
people in both countries were arrested & charged for merely selling organic apricot seeds, a carrier of unapproved vitamin B17. (laetrile).


OUT
OF
CONTROL
THE UNITED STATES OF AMERICA
E PLURIBUS UNUM
The Story of the Reagan Administration's Secret War in Nicaragua, the Illegal Arms Pipeline, and the Contra Drug Connection
LESLIE COCKBURN

The Contras, Cocaine, and Covert Operations

National Security Archive Electronic Briefing Book No. 2

For more information contact:
202/994-7000 or nsarchiv@gwu.edu

Washington, D.C. – An August, 1996, series in the *San Jose Mercury News* by reporter Gary Webb linked the origins of crack cocaine in California to the contras, a guerrilla force backed by the Reagan administration that attacked Nicaragua's Sandinista government during the 1980s. Webb's series, "The Dark Alliance," has been the subject of intense media debate, and has focused attention on a foreign policy drug scandal that leaves many questions unanswered.

This electronic briefing book is compiled from declassified documents obtained by the National Security Archive, including the notebooks kept by NSC aide and Iran-contra figure Oliver North, electronic mail messages written by high-ranking Reagan administration officials, memos detailing the contra war effort, and FBI and DEA reports. The documents demonstrate official knowledge of drug operations, and collaboration with and protection of known drug traffickers. Court and hearing transcripts are also included.

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Documentation of Official U.S. Knowledge of Drug Trafficking and the Contras

The National Security Archive obtained the hand-written notebooks of Oliver North, the National Security Council aide who helped run the contra war and other Reagan administration covert operations, through a Freedom of Information Act lawsuit filed in 1989. The notebooks, as well as declassified memos sent to North, record that North was repeatedly informed of contra ties to drug trafficking.

In his entry for August 9, 1985, North summarizes a meeting with Robert Owen ("Rob"), his liaison with the contras. They discuss a plane used by Mario Calero, brother of Adolfo Calero, head of the FDN, to transport supplies from New Orleans to contras in Honduras. North writes: "Honduran DC-6 which is being used for runs out of New Orleans is probably being used for drug runs into U.S." As Lorraine Adams reported in the October 22, 1994 *Washington Post*, there are no records that corroborate North's later assertion that he passed this intelligence on drug trafficking to the U.S. Drug Enforcement Administration.

In a July 12, 1985 entry, North noted a call from retired Air Force general Richard Secord in which the two discussed a Honduran arms warehouse from which the contras planned to purchase weapons. (The contras did eventually buy the arms, using money the Reagan administration secretly raised from Saudi Arabia.) According to the notebook, Secord told North that "14 M to finance [the arms in the warehouse] came from drugs."

An April 1, 1985 memo from Robert Owen (code-name: "T.C." for "The Courier") to Oliver North (code-name: "The Hammer") describes contra operations on the Southern Front. Owen tells North that FDN leader Adolfo Calero (code-name: "Sparkplug") has picked a new Southern Front commander, one of the former captains to Eden Pastora who has been paid to defect to the FDN. Owen reports that the officials in the new Southern Front FDN units include "people who are questionable because of past indiscretions," such as José Robelo, who is believed to have "potential involvement with drug running" and Sebastian Gonzalez, who is "now involved in drug running out of Panama."

On February 10, 1986, Owen ("TC") wrote North (this time as "BG," for "Blood and Guts") regarding a plane being used to carry "humanitarian aid" to the contras that was previously used to transport drugs. The plane belongs to the Miami-based company Vortex, which is run by Michael Palmer, one of the largest marijuana traffickers in the United States. Despite Palmer's long history of drug smuggling, which would soon lead to a Michigan indictment on drug charges, Palmer receives over $300,000.00 from the Nicaraguan Humanitarian Aid Office (NHAO) -- an office overseen by Oliver North, Assistant Secretary of State for Inter-American Affairs Elliott Abrams, and CIA officer Alan Fiers -- to ferry supplies to the contras.

State Department contracts from February 1986 detail Palmer's work to transport material to the contras on behalf of the NHAO.

Evidence that NSC Staff Supported Using Drug Money to Fund the Contras

In 1987, the Senate Subcommittee on Narcotics, Terrorism and International Operations, led by Senator John Kerry, launched an investigation of allegations arising from reports, more than a decade ago, of contra-drug links. One of the incidents examined by the "Kerry Committee" was an effort to divert drug money from a counternarcotics operation to the contra war.

On July 28, 1988, two DEA agents testified before the House Subcommittee on Crime regarding a sting operation

conducted against the Medellin Cartel. The two agents said that in 1985 Oliver North had wanted to take $1.5 million in Cartel bribe money that was carried by a DEA informant and give it to the contras. DEA officials rejected the idea.

The Kerry Committee report concluded that "senior U.S. policy makers were not immune to the idea that drug money was a perfect solution to the Contras' funding problems."

U.S. Officials and Major Traffickers

Manuel Noriega

In June, 1986, the New York Times published articles detailing years of Panamanian leader Manuel Noriega's collaboration with Colombian drug traffickers. Reporter Seymour Hersh wrote that Noriega "is extensively involved in illicit money laundering and drug activities," and that an unnamed White House official "said the most significant drug running in Panama was being directed by General Noriega." In August, Noriega, a long-standing U.S. intelligence asset, sent an emissary to Washington to seek assistance from the Reagan administration in rehabilitating his drug-stained reputation.

Oliver North, who met with Noriega's representative, described the meeting in an August 23, 1986 e-mail message to Reagan national security advisor John Poindexter. "You will recall that over the years Manuel Noriega in Panama and I have developed a fairly good relationship," North writes before explaining Noriega's proposal. If U.S. officials can "help clean up his image" and lift the ban on arms sales to the Panamanian Defense Force, Noriega will "'take care of' the Sandinista leadership for us."

North tells Poindexter that Noriega can assist with sabotage against the Sandinistas, and suggests paying Noriega a million dollars -- from "Project Democracy" funds raised from the sale of U.S. arms to Iran -- for the Panamanian leader's help in destroying Nicaraguan economic installations.

The same day Poindexter responds with an e-mail message authorizing North to meet secretly with Noriega. "I have nothing against him other than his illegal activities," Poindexter writes.

On the following day, August 24, North's notebook records a meeting with CIA official Duane "Dewey" Clarridge on Noriega's overture. They decided, according to this entry, to "send word back to Noriega to meet in Europe or Israel."

The CIA's Alan Fiers later recalls North's involvement with the Noriega sabotage proposal. In testimony at the 1992 trial of former CIA official Clair George, Fiers describes North's plan as it was discussed at a meeting of the Reagan administration's Restricted Interagency Group: "[North] made a very strong suggestion that . . . there needed to be a resistance presence in the western part of Nicaragua, where the resistance did not operate. And he said, 'I can arrange to have General Noriega execute some insurgent -- some operations there -- sabotage operations in that area. It will cost us about $1 million. Do we want to do it?' And there was significant silence at the table. And then I recall I said, 'No. We don't want to do that.'"

Senior officials ignored Fiers' opinion. On September 20, North informed Poindexter via e-mail that "Noriega wants to meet me in London" and that both Elliott Abrams and Secretary of State George Shultz support the initiative. Two days later, Poindexter authorized the North/Noriega meeting.

North's notebook lists details of his meeting with Noriega, which took place in a London hotel on September 22. According to the notes, the two discussed developing a commando training program in Panama, with Israeli support, for the contras and Afghani rebels. They also spoke of sabotaging major economic targets in the Managua area, including an airport, an oil refinery, and electric and telephone systems. (These plans were apparently aborted when the Iran-Contra scandal broke in November 1986.)

José Bueso Rosa

Reagan administration officials interceded on behalf of José Bueso Rosa, a Honduran general who was heavily involved with the CIA's contra operations and faced trial for his role in a massive drug shipment to the United States. In 1984 Bueso and co-conspirators hatched a plan to assassinate Honduran President Roberto Suazo Córdoba; the plot was to be financed with a $40 million cocaine shipment to the United States, which the FBI intercepted in Florida.

Declassified e-mail messages indicate that Oliver North led the behind-the-scenes effort to seek leniency for Bueso . The messages record the efforts of U.S. officials to "cabal quietly" to get Bueso off the hook, be it by "pardon, clemency, deportation, [or] reduced sentence." Eventually they succeeded in getting Bueso a short sentence in "Club Fed," a white collar prison in Florida.

The Kerry Committee report reviewed the case, and noted that the man Reagan officials aided was involved in a conspiracy that the Justice Department deemed the "most significant case of narco-terrorism yet discovered."

FBI/DEA Documentation

In February 1987 a contra sympathizer in California told the FBI he believed FDN officials were involved in the drug trade. Dennis Ainsworth, a Berkeley-based conservative activist who had supported the contra cause for years, gave a lengthy description of his suspicions to FBI agents. The bureau's debriefing says that Ainsworth agreed to be interviewed because "he has certain information in which he believes the Nicaraguan 'Contra' organization known as FDN (Frente Democrático Nacional) has become more involved in selling arms and cocaine for personal gain than in a military effort to overthrow the current Nicaraguan Sandinista Government." Ainsworth informed the FBI of his extensive contacts with various contra leaders and backers, and explained the basis for his belief that members of the FDN were trafficking in drugs.

A DEA report of February 6, 1984 indicates that a central figure in the *San Jose Mercury News* series was being tracked by U.S. law enforcement officials as early as 1976, when a DEA agent "identified Norwin MENESES-Canterero as a cocaine source of supply in Managua, Nicaragua." Meneses, an associate of dictator Anastasio Somoza who moved to California after the Nicaraguan revolution in 1979, was an FDN backer and large-scale cocaine trafficker.

Testimony of Fabio Ernesto Carrasco, 6 April 1990

On October 31, 1996, the *Washington Post* ran a follow up story to the *San Jose Mercury News* series titled "CIA, Contras and Drugs: Questions on Links Linger." The story drew on court testimony in 1990 of Fabio Ernesto Carrasco, a pilot for a major Columbian drug

smuggler named George Morales. As a witness in a drug trial, Carrasco testified that in 1984 and 1985, he piloted planes loaded with weapons for contras operating in Costa Rica. The weapons were offloaded, and then drugs stored in military bags were put on the planes which flew to the United States. "I participated in two [flights] which involved weapons and cocaine at the same time," he told the court. Carrasco also testified that Morales provided "several million dollars" to Octaviano Cesar and Adolfo "Popo" Chamorro, two rebel leaders working with the head of the contras' southern front, Eden Pastora. *The Washington Post* reported that Chamorro said he had called his CIA control officer to ask if the contras could accept money and arms from Morales, who was at the time under indictment for cocaine smuggling. "They said [Morales] was fine," Chamorro told the *Post*.

National Security Archive Analysis and Publications

- Peter Kornbluh's Testimony at California Congressional Inquiry (19 October 1996)
- "Crack, Contras, and the CIA: The Storm Over 'Dark Alliance,'" from *Columbia Journalism Review* (January/February 1997)
- "CIA's Challenge in South Central," from the *Los Angeles Times (15 November 1996)*
- "The Paper Trail to the Top," from the *Baltimore Sun (17 November 1996)*
- *White House E-Mail: The Top Secret Computer Messages the Reagan/Bush White House Tried to Destroy*
- *The Iran-Contra Scandal: the Declassified History*

Partnership for a Drug-Wrecked America

Mike Males
Published: October 17, 2013

The Partnership at Drugfree.org has always felt free to lie—blatantly, openly, stupidly—about drugs. In fact, lying to obscure the realities of drug abuse in order to protect powerful interests and constituencies is the reason the Partnership exists.

Called the Partnership for a Drug-Free America when it formed in 1985, it was funded by alcohol, tobacco, and pharmaceutical and big-advertising interests worried that escalating War on Drugs fervor might impact its profits. Big funders then included American Brands (Jim Beam whiskey), Philip Morris (Marlboro and Virginia Slims cigarettes, Miller beer), Anheuser Busch (Budweiser, Michelob, Busch beer), R.J. Reynolds (Camel, Salem, Winston cigarettes), and Big Pharma firms like Bristol Meyers-Squibb, Merck & Company and Proctor & Gamble.

In 1997, the Partnership abstained from legal-drug funding and now gets its bucks from the drug-war cabal, which is hardly more honest.

Lying about critical issues in gigantic advertising campaigns has been the Partnership's motif. In a 1989 ad, the Partnership mispresented the electroencephalograph of a comatose patient as "the brain waves of a marijuana smoker." Another Partnership ad declared that 10 million of the 15 million Americans who used cocaine had died from the drug and the remaining 5 million required medical help, wildly excessive figures exposed as fabricated in an inquiry from Scientific American. And of course, there was the Partnership's famous egg-frying "this is your brain on drugs" idiocy.

The Partnership is the latest in America's long history of phony lobbies—the Office of National Drug Control Policy (ONDCP) is the White House branch—that revel in misinformation and misdirected policies that perpetuate the social crises they claim to be attacking because they tacitly profit from making them worse.

While the Partnership for "Drug Free" (if this blog had a laugh track, cue it now) America was bankrolled by magnates whose legal-drug products caused hundreds of thousands of deaths annually, the new Partnership can now boast of a similar achievement: during its quarter-century of multi-billion-dollar ad campaigns, deaths from illicit drugs soared by the tens of thousands and now also constitute a major public health crisis.

When the War on Drugs, pivotally shaped by Partnership ideas and lobbying, was launched in the mid-1980s, Centers for Disease Control tabulations showed around 9,500 Americans died every year from illicit drug abuse. Today, after three decades of anti-drug warring, tens of millions of arrests, and lavishly funded Partnership crusades, illicit-drug abuse deaths have quadrupled to nearly 40,000 and now kill more Americans than guns or traffic crashes do. Mission accomplished!

Credible United Nations estimates now indicate the United States, with 5% of the world's population, now accounts for fully 60% of consumption of illegal drugs worldwide and suffer drug tolls far in excess of any other country. The latest World Health Association figures show Americans are 6 times more likely to die from drug abuse than citizens in comparable Western and Latin American countries.

Social catastrophes such as a widespread, lethal drug abuse epidemic is the ideal outcome for American drug-war interests led by ONDCP and the Partnership, the predictable result of their stubborn, punitively ineffective "harm maximization" strategies sensible nations have long rejected.

The Partnership, in particular, aided and abetted the rise of the deadliest illicit-drug abuse pandemic in American history with good old market research delineating how to use misinformation and emotional images to divert attention from real drug issues. Through continual surveys and focus groups, the Partnership honed a set of pleasing lies blaming drug problems on an unpopular minority—a long proven tactic, dating back to the Chinese-opium, Negroes-cocaine, and Mexicans-marihuana days, and now vilifying young people—as the ideal means of popularizing its message with politicians, funders, and the public.

For example, the Partnership's latest "Not in My House" campaign (with funding from Big Pharma) ignores the drug-related deaths of tens of thousands of Americans—inconveniently centered in middle-aged whites who abuse pharmaceuticals—in favor of shrewd, candied-up PR ads soothing that the big problem is just teenagers pilfering pills from their parents' medicine cabinets. The ad notes that pharmaceutical abuse has become the nation's worst drug crisis, then pretends the crisis is teens (a couple of hundred of whom died in 2011) while ignoring the middle-aged epidemic (more than 10,000 deaths).

A truthful and useful campaign—far too rough for the wimpy Partnership and ONDCP authorities—would be the opposite; to advise kids how to deal with parents and elders whose abuse of illegal pharmaceuticals is disrupting families and communities and is the real predictor of

later drug abuse by their kids. But such an honest campaign might really help reduce drug abuse, or at least describe it accurately to inform effective policy—results that would horrify entrenched drug-war interests.

During the 30-year Drug War and Partnership reign, drug abuse has exploded to generate hundreds of thousands of addicts driving record fatalities, intractable crime, and—except where sensible reforms such as California's Proposition 36 have intervened—are filling prisons in record numbers. This is not an accident.

Drug War lobbies again and again roll out distracting, emotional crusades vilifying teenagers to quash efforts to establish genuine, research-informed, effective drug abuse amelioration measures. Maintaining drug abuse while pretending to "war on drugs" serves a myriad of powerful industries, including the Partnership. This is why their seemingly stupid ads like "Not in My House" must be taken seriously as evidencing yet again how privatized social policy driven by interest groups profiting from misery continues to saddle the U.S. with the worst social epidemics of any modern society.

Kennedy

In 1971 President Richard Nixon declared war on Drugs. He proclaimed,
“America's public enemy number one in the United States is drug abuse. In order to fight and defeat this enemy, it is necessary to wage a new, all-out offensive.”

This helped bring in “free enterprise” -the umbrella the drug business hides under. Billions and billions go “missing” and are unaccounted for. They go through Shanghai Banks with shipments of drugs through Hong Kong. This has been the case since the Naphtali tribe, as we have seen. We must stop kidding ourselves that drugs are a problem. There is no drug problem besides the Elite. They create the drugs, make most of their income on drugs, and always have, particularly Opium, and disperse it through the United States and all around the world. That is why they have such a fear of legalizing Marijuana. Marijuana been proven by them to be a drug which produces free thought and an inability to stay brainwashed. If they do, they lose a lot of their revenue, even though it would be regulated and taxed. The improbabilities they face of releasing this to society as acceptable, are too numerous. What is everyone was able to be woken up from their mental control? The Elite want their freedoms uninhibited.
Samuel Morse wrote:
"As long as this country maintains its drug traffic, there is not the slightest possibility
that it will ever become a military threat, since the habit saps the vitality of the nation." in 1835 under the pen-name Brutus. This rings true as most quotes do. So, in all likelihood, whatever it is you are addicted to it is not necessarily your fault since the government's goal with drugs has been to get you addicted to them in order to control you easier. However, you have the control and the power to stop. The war on drugs is simply a war on you and your free will.

Onassis & Li

Let's continue with the families, now that we are almost caught up to current day.
We have learned a bit about the Onassis family already but let's look closer. Aristotle Socrates Onassis married widow Jackie Kennedy. Jaqueline Bouvier Kennedy Onassis' father was a member a club that mirrored the Order of the Garter. Her mother was Senator Thomas Gore's daughter. Needless to say, she was well connected before her marriage. Aristotle died in 1975 and, unfortunately, his heir had died two years before. His daughter was found dead in a bathtub a few years later and his illegitimate son, who he had with his mistress, Anna Maria Sofia Cecilia Kalogeropoulou Callas was also "dead" on the day of his birth, March 30, 1960. It's an interesting note as it is the first child of the families that was born in the same time Obama is said to have been born. Especially with both parents being Turkish-Greek, which could easily be Obama's true heritage. Let's look further into the line.
Aristotle Socrates Onassis (aka Ari, Aristotelis Sokratis) was born January 20, 1906 in Karatass, Smyrna (also Smirne), Greece (now Turkey) and moved to Buenos Aires, France, and Greece. If you take a look at him it is clear to see he was "top notch". We will note a few things. We have already shown the link of Onassis to the Mafia and traced them back to it's creation by Mazzini in Italy. He, being the new godfather, was deeply involved in opium trade through the Golden Triangle.
About this time US State Department issued a plan to disarm all nations and arm the United Nations. Document Number 7277 is entitled Freedom From War: The Program for General and Complete Disarmament in a Peaceful World. It details a three stage plan (they like things in three's), the final stage in which "no state would have the military power to challenge the progressively strengthened UN Peace Force."
Once Joseph died, JFK and Bobby were finally “free” to attack the elite. They arrested Wally Bird, who owned the Air Thailand (the line used for shipping Onassis opium under CIA's Air Opium program), arrested Mafiaman Jimmy Hoffa, and declared the $73 million in fake Hughes land liens, deposited them with SF Bank of America, which declared them forgeries.
You see, in March 1957, a small group had come up with a very detailed plan on how to acquire their own airline in order to smuggle even more opium around the world. Unable to use commercial airlines in USA, any longer, they noticed Howard Hughes. His stonewall for the commercial control over his airline made him the perfect target. The plan fell together and Onassis was the executor.
He has Howard Hughes kidnapped, as we touched on before, from his bungalow at the Beverly Hills Hotel, using Hughes' own men. Hughes is shot full of heroin for thirty days and later dragged to a cell on Onassis' island, Skerpiog. Onassis now has more power in the US with the Hughes Empire, already controlled Nixon, and other Hughes purchased politicians. Hughes' double played him out for years. When JFK found out about this, once his brother had died, he rose up.
Onassis put up an elite team of assassins to murder Castro since JFK wasn't going with the plan. JFK and Bobby set up a "justice team" and arrested the assassins. Onassis is beyond enraged, as you can imagine, and turns the assassins onto JFK. JFK created the "Group of 40". JFK's assassination was going to take place as a triple execution, together with Diem and Nhu in Vietnam. Diem and Nhu were whacked as scheduled. This is not the only thing going on with JFK that would have gotten him murdered. If one plot didn't work, the next wave would come.

Another person who found out about the earlier deal between Joseph Kennedy and Onassis, Senator Estes Kefauver,planned a speech on the Senate floor exposing the Mafia operations and the members: Onassis, Kennedy, Eugene Meyer, Lansky, Roosevelt, etc. He ate a piece of Apple Pie laced with sodium morphate (poison), and had a heart attack on the Senate floor. There is no such thing as freedom of speech.

ALTERNATIVE DRUGS & MEDICINE

The Marijuana Conspiracy - The Real Reason Hemp is Illegal
by Doug Yurchey, June 15, 2005

NOTE TO THE READER: Words and sentences within this type of brackets [ ] are not part of the original article!

And I will raise up for them a plant of renown, and they shall be no more consumed with hunger in the land.-- *Ezekiel 34/29*

THE REAL REASON CANNABIS HAS BEEN OUTLAWED HAS NOTHING TO DO WITH ITS EFFECTS ON THE MIND AND BODY.

MARIJUANA is DANGEROUS. Pot is NOT harmful to the human body or mind. Marijuana does NOT pose a threat to the general public. Marijuana is very much a danger to the oil companies, alcohol, tobacco **industries** and a large number of chemical corporations. Various big businesses, with plenty of dollars and influence, have suppressed the truth from the people.

The truth is if marijuana was utilized for its vast array of commercial products, it would create an industrial atomic bomb! Entrepreneurs have not been educated on the product potential of pot. The super rich have conspired to spread misinformation about an extremely versatile plant that, if used properly, would ruin their companies.

Where did the word 'marijuana' come from? In the mid 1930s, the M-word was created to tarnish the good image and phenomenal history of the hemp plant...as you will read. The facts cited here, with references, are generally verifiable in the Encyclopedia Britannica which was printed on hemp paper for 150 years:

* All schoolbooks were made from hemp or flax paper until the 1880s; Hemp Paper Reconsidered, Jack Frazier, 1974.
* It was LEGAL TO PAY TAXES WITH HEMP in America from 1631 until the early 1800s; LA Times, Aug. 12, 1981.
* REFUSING TO GROW HEMP in America during the 17th and 18th Centuries WAS AGAINST THE LAW! You could be jailed in Virginia for refusing to grow hemp from 1763 to 1769; Hemp in Colonial Virginia, G. M. Herdon.

* George Washington, Thomas Jefferson and other founding fathers GREW HEMP; Washington and Jefferson Diaries. Jefferson smuggled hemp seeds from China to France then to America.
* Benjamin Franklin owned one of the first paper mills in America and it processed hemp. Also, the War of 1812 was fought over hemp. Napoleon wanted to cut off Moscow's export to England; Emperor Wears No Clothes, Jack Herer.
* For thousands of years, 90% of all ships' sails and rope were made from hemp. The word 'canvas' is Dutch for cannabis; Webster's New World Dictionary.
* 80% of all textiles, fabrics, clothes, linen, drapes, bed sheets, etc. were made from hemp until the 1820s with the introduction of the cotton gin.
* The first Bibles, maps, charts, Betsy Ross's flag, the first drafts of the Declaration of Independence and the Constitution were made from

hemp; U.S. Government Archives.
* The first crop grown in many states was hemp. 1850 was a peak year for Kentucky producing 40,000 tons. Hemp was the largest cash crop until the 20th Century; State Archives.
* Oldest known records of hemp farming go back 5000 years in China, although hemp industrialization probably goes back to ancient Egypt.
* Rembrants, Gainsboroughs, Van Goghs as well as most early canvas paintings were principally painted on hemp linen.
* In 1916, the U.S. Government predicted that by the 1940s all paper would come from hemp and that no more trees need to be cut down. Government studies report that 1 acre of hemp equals 4.1 acres of trees. Plans were in the works to implement such programs; Department of Agriculture
* Quality paints and varnishes were made from hemp seed oil until 1937. 58,000 tons of hemp seeds were used in America for paint products in 1935; Sherman Williams Paint Co. testimony before Congress against the 1937 Marijuana Tax Act.
* Henry Ford's first Model-T was built to run on hemp gasoline and the CAR ITSELF WAS CONTRUCTED FROM HEMP! On his large estate, Ford was photographed among his hemp fields. The car, 'grown from the soil,' had hemp plastic panels whose impact strength was 10 times stronger than steel; Popular Mechanics, 1941.
* Hemp called 'Billion Dollar Crop.' It was the first time a cash crop had a business potential to exceed a billion dollars; Popular Mechanics, Feb., 1938.
* Mechanical Engineering Magazine (Feb. 1938) published an article entitled 'The Most Profitable and Desirable Crop that Can be Grown.' It stated that if hemp was cultivated using 20th Century technology, it would be the single largest agricultural crop in the U.S. and the rest of the world.
The following information comes directly from the United States Department of Agriculture's 1942 14-minute film encouraging and instructing 'patriotic American farmers' to grow 350,000 acres of hemp each year for the war effort:
'...(When) Grecian temples were new, hemp was already old in the service of mankind. For thousands of years, even then, this plant had been grown for cordage and cloth in China and elsewhere in the East. For centuries prior to about 1850, all the ships that sailed the western seas were rigged with hempen rope and sails. For the sailor, no less than the hangman, hemp was indispensable...
...Now with Philippine and East Indian sources of hemp in the hands of the Japanese...American hemp must meet the needs of our Army and Navy as well as of our industries...
...the Navy's rapidly dwindling reserves. When that is gone, American hemp will go on duty again; hemp for mooring ships; hemp for tow lines; hemp for tackle and gear; hemp for countless naval uses both on ship and shore. Just as in the days when Old Ironsides sailed the seas victorious with her hempen shrouds and hempen sails. Hemp for victory!'
Certified proof from the Library of Congress; found by the research of Jack Herer, refuting claims of other government agencies that the 1942 USDA film 'Hemp for Victory' did not exist.

Hemp cultivation and production do not harm the environment.

The USDA Bulletin #404 concluded that hemp produces 4 times as much pulp with at least 4 to 7 times less pollution. From Popular Mechanics, Feb. 1938:
'It has a short growing season...It can be grown in any state...The long roots penetrate and break the soil to leave it in perfect condition for the next year's crop. The dense shock of leaves, 8 to 12 feet above the ground, chokes out weeds.
...hemp, this new crop can add immeasurably to American agriculture and industry.'
In the 1930s, innovations in farm machinery would have caused an industrial revolution when applied to hemp. This single resource could have created millions of new jobs generating thousands of quality products. Hemp, if not made illegal, would have brought America out of the Great Depression.
William Randolph Hearst (Citizen Kane) and the Hearst Paper Manufacturing Division of Kimberly Clark owned vast acreage of timberlands. The Hearst Company supplied most paper products. Patty Hearst's grandfather, a destroyer of nature for his own personal profit, stood to

lose billions because of hemp.
In 1937, Dupont patented the processes to make plastics from oil and coal. Dupont's Annual Report urged stockholders to invest in its new petrochemical division. Synthetics such as plastics, cellophane, celluloid, methanol, nylon, rayon, Dacron, etc., could now be made from oil. Natural hemp industrialization would have ruined over 80% of Dupont's business.

THE CONSPIRACY

Andrew Mellon became Hoover's Secretary of the Treasury and Dupont's primary investor. He appointed his future nephew-in-law, Harry J. Anslinger, to head the Federal Bureau of Narcotics and Dangerous Drugs.
Secret meetings were held by these financial tycoons. Hemp was declared dangerous and a threat to their billion dollar enterprises. For their dynasties to remain intact, hemp had to go. These men took an obscure Mexican slang word: 'marihuana' and pushed it into the consciousness of America.

MEDIA MANIPULATION

A media blitz of 'yellow journalism' raged in the late 1920s and 1930s. Hearst's newspapers ran stories emphasizing the horrors of marihuana. The menace of marihuana made headlines. Readers learned that it was responsible for everything from car accidents to loose morality.
Films like 'Reefer Madness' (1936), 'Marihuana: Assassin of Youth' (1935) and 'Marihuana: The Devil's Weed' (1936) were propaganda designed by these industrialists to create an enemy. Their purpose was to gain public support so that anti-marihuana laws could be passed.
Examine the following quotes from 'The Burning Question' aka REEFER MADNESS:

- a violent narcotic.
- acts of shocking violence.
- incurable insanity.
- soul-destroying effects.
- under the influence of the drug he killed his entire family with an ax.
- more vicious, more deadly even than these soul-destroying drugs (heroin, cocaine) is the menace of marihuana!

Reefer Madness did not end with the usual 'the end.' The film concluded with these words plastered on the screen: TELL YOUR CHILDREN.
In the 1930s, people were very naive; even to the point of ignorance. The masses were like sheep waiting to be led by the few in power. They did not challenge authority. If the news was in print or on the radio, they believed it had to be true. They told their children and their children grew up to be the parents of the baby-boomers.
On April 14, 1937, the Prohibitive Marihuana Tax Law or the bill that outlawed hemp was directly brought to the House Ways and Means Committee. This committee is the only one that can introduce a bill to the House floor without it being debated by other committees. The Chairman of the Ways and Means, Robert Doughton, was a Dupont supporter. He insured that the bill would pass Congress.
Dr. James Woodward, a physician and attorney, testified too late on behalf of the American Medical Association. He told the committee that the reason the AMA had not denounced the Marihuana Tax Law sooner was that the Association had just discovered that marihuana was hemp.
Few people, at the time, realized that the deadly menace they had been reading about on Hearst's front pages was in fact passive hemp. The AMA understood cannabis to be a MEDICINE found in numerous healing products sold over the last hundred years.
In September of 1937, hemp became illegal. The most useful crop known became a drug and our planet has been suffering ever since.
Congress banned hemp because it was said to be the most violence-causing drug known. Anslinger, head of the Drug Commission for 31 years, promoted the idea that marihuana made users act extremely violent. In the 1950s, under the Communist threat of McCarthyism, Anslinger now said the exact opposite. Marijuana will pacify you so much that soldiers would not want to fight.
Today, our planet is in desperate trouble. Earth is suffocating as large tracts of rain forests disappear. Pollution, poisons and chemicals are killing people. These great problems could be reversed if we industrialized hemp. Natural biomass could provide all of the planet's energy needs that are currently supplied by fossil fuels. We have consumed 80% of our oil and gas reserves. We need a renewable resource. Hemp could be the solution to soaring gas prices.

THE WONDER PLANT

Hemp has a higher quality fiber than wood fiber. Far fewer caustic chemicals are required to make paper from hemp than from trees. Hemp paper does not turn yellow and is very durable. The plant grows quickly to maturity in a season where trees take a lifetime.

ALL PLASTIC PRODUCTS SHOULD BE MADE FROM HEMP SEED OIL. Hempen plastics are biodegradable! Over time, they would break down and not harm the environment. Oil-based plastics, the ones we are very familiar with, help ruin nature; they do not break down and will do great harm in the future. The process to produce the vast array of natural (hempen) plastics will not ruin the rivers as Dupont and other petrochemical companies have done. Ecology does not fit in with the plans of the Oil Industry and the political machine. Hemp products are safe and natural.

MEDICINES SHOULD BE MADE FROM HEMP. We should go back to the days when the AMA supported cannabis cures. 'Medical Marijuana' is given out legally to only a handful of people while the rest of us are forced into a system that relies on chemicals. Pot is only healthy for the human body.

WORLD HUNGER COULD END. A large variety of food products can be generated from hemp. The seeds contain one of the highest sources of protein in nature. ALSO: They have two essential fatty acids that clean your body of cholesterol. These essential fatty acids are not found anywhere else in nature! Consuming pot seeds is the best thing you could do for your body. Eat uncooked hemp seeds.

CLOTHES SHOULD BE MADE FROM HEMP. Hemp clothing is extremely strong and durable over time. You could hand clothing, made from pot, down to your grandchildren. Today, there are American companies that make hemp clothing; usually 50% hemp. Hemp fabrics should be everywhere. Instead, they are almost underground. Superior hemp products are not allowed to advertise on fascist television. Kentucky, once the top hemp producing state, made it ILLEGAL TO WEAR hemp clothing! Can you imagine being thrown into jail for wearing quality jeans?

The world is crazy...but that does not mean you have to join the insanity. Get together. Spread the news. Tell people, and that includes your children, the truth. Use hemp products. Eliminate the word 'marijuana.' Realize the history that created it. Make it politically incorrect to say or print the M-word. Fight against the propaganda (designed to favor the agenda of the super rich) and the bullshit. Hemp must be utilized in the future. We need a clean energy source to save our planet. INDUSTRIALIZE HEMP!

The liquor, tobacco and oil companies fund more than a million dollars a day to Partnership for a Drug-Free America and other similar agencies. We have all seen their commercials. Now, their motto is: ‘It's more dangerous than we thought.’ Lies from the powerful corporations, that began with Hearst, are still alive and well today.

The brainwashing continues. Now, the commercials say: If you buy a joint, you contribute to murders and gang wars. The latest anti-pot commercials say: If you buy a joint...you are promoting TERRORISM! The new enemy (terrorism) has paved the road to brainwash you any way THEY see fit.

There is only one enemy; the friendly people you pay your taxes to; the war-makers and nature destroyers. With your funding, they are killing the world right in front of your eyes. HALF A MILLION DEATHS EACH YEAR ARE CAUSED BY TOBACCO. HALF A MILLION DEATHS EACH

YEAR ARE CAUSED BY ALCOHOL. NO ONE HAS EVER, EVER DIED FROM SMOKING POT!! In the entire history of the human race, not one death can be attributed to cannabis. Our society has outlawed grass but condones the use of the KILLERS: TOBACCO and ALCOHOL. Hemp should be declassified and placed in DRUG stores to relieve stress. Hardening and constriction of the arteries are bad; but hemp usage actually enlarges the arteries...which is a healthy condition. We have been so conditioned to think that: Smoking is harmful. That is NOT the case for passive pot.

Ingesting THC, hemp's active agent, has a positive effect; relieving asthma and glaucoma. A joint tends to alleviate the nausea caused by chemotherapy. You are able to eat on hemp. This is a healthy state of being.

(One personal note: During the pregnancy of my wife, she was having some difficulty gaining weight. We were in the hospital. A nurse called us to one side and said: 'Off the record, if you smoke pot...you'd get something called the munchies and you'll gain weight.' I swear that is a true story). [Using Medifast coupons to counteract the results of the weight gain later on is a different story.]

The stereotype for a pothead is similar to a drunk, bubble-brain. Yet, the truth is one's creative abilities can be enhanced under its influence. The perception of time slightly slows and one can become more sensitive. You can more appreciate all arts; be closer to nature and generally FEEL more under the influence of cannabis. It is, in fact, the exact opposite state of mind and body as the drunken state. You can be more aware with pot.

The pot plant is an ALIEN plant. There is physical evidence that cannabis is not like any other plant on this planet. One could conclude that it was brought here for the benefit of humanity. Hemp is the ONLY plant where the males appear one way and the females appear very different, physically! No one ever speaks of males and females in regard to the plant kingdom because plants do not show their sexes; except for cannabis. To determine what sex a certain, normal, Earthly plant is: You have to look internally, at its DNA. A male blade of grass (physically) looks exactly like a female blade of grass. The hemp plant has an intense sexuallity. Growers know to kill the males before they fertilize the females. Yes, folks...the most potent pot comes from 'horny females.'

The reason this amazing, very sophisticated, ET plant from the future is illegal has nothing to do with how it physically affects us.....

...POT IS ILLEGAL BECAUSE BILLIONAIRES WANT TO REMAIN BILLIONAIRES!

ps: I think the word 'DRUGS' should not be used as an umbrella-word that covers all chemical agents. Drugs have come to be known as something BAD. Are you aware there are LEGAL drugstores?! Yep, in every city. Unbelievable. Each so-called drug should be considered individually. Cannabis is a medicine and not a drug. We should DARE to speak the TRUTH no matter what the law is.

Also read Doug Yurchey's follow-up article, "Marijuana Conspiracy - the Sequel" here at the Illuminati News website.

Comments and questions are welcome: dugko@surfside.net

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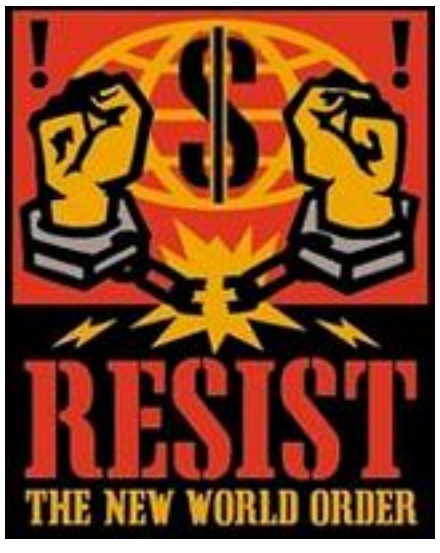

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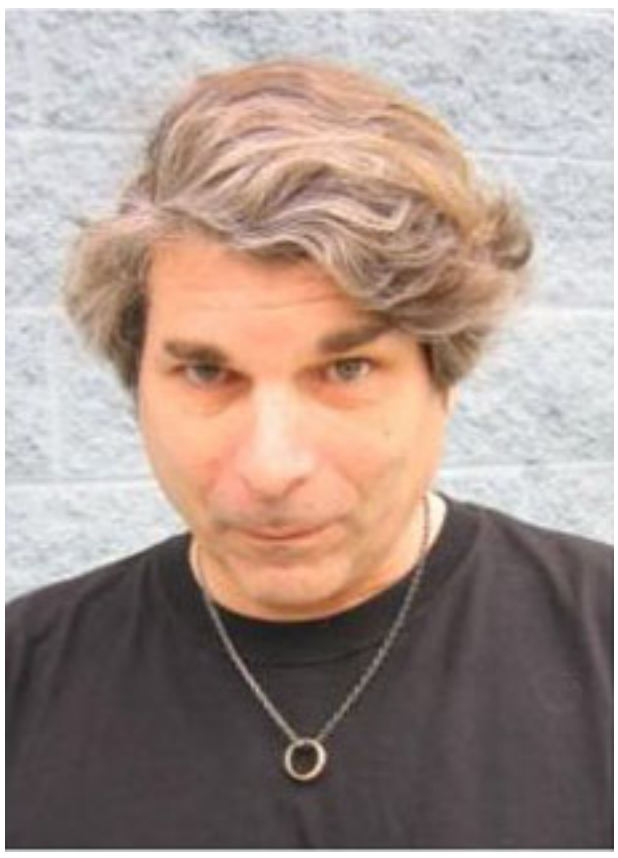
ALTERNATIVE DRUGS & MEDICINE

Marijuana Conspiracy - the Sequel

by Doug Yurchey, May 29, 2007



In 2005, this writer penned 'The Marijuana Conspiracy - The Real Reason Hemp is Illegal' and it became (by far) my most popular article. Translated into Italian, French, Spanish, Russian, Swedish, Bulgarian and even Japanese, the responses were overwhelming. Its premise was 'marijuana' became illegal NOT because it was a danger to the mind and body. No, the real reason is that Big Business wants us to use petrochemicals and fossil fuels. They have little interest in NATURAL solutions. THEY have suppressed the truth concerning cannabis and purposely created the 'menace of marijuana.' Why? So they remain high profiteers while destroying our environment in the process.

Once people read the news of what has been kept from them, the truth, the reactions were outrage and disbelief at our federal authorities. Hemp used properly for all of its INDUSTRIAL purposes, as it was always meant to be utilized, could...

SAVE THE WORLD! We need not cut down another tree. We need not have to rely on Oil Companies anymore.

So many people wrote to me and said the suppression of hemp is 'insane.' Surely, governments know the potential of pot. They understand how cannabis hemp could solve the energy crisis; they know biodegradable plastics could be produced from the hemp plant; they realize it would be a safer, cleaner world filled with (inexpensive) quality products...if only we were federally given the green light to INDUSTRIALIZE HEMP! Governments are well aware of the MEDICINAL value for 'weed.' New cloths, papers and plastics would be super durable. They could easily create nutritional FOOD products for a starving world. BUT...when did a better mousetrap ever see the light of day?

Now, with soaring gas prices bleeding us for every dollar inflating all prices, we need a cheap (renewable) fuel and energy source more than ever. Imagine hemp gas. The price of it would not be sky high since we have a limitless supply of hempen energy. Federal (guarded) hemp fields could be farmed and directed to produce a cheap/clean gasoline. Hemp fibers and oil were utilized during WWII for rope-moorings, parachutes and lubrication. Why not use hemp gasoline NOW when we are at another critical point in time? Why does hemp technology remain clandestine?

Why is there a war in the Middle East over oil? Do you really think it's a coincidence that George Bush is an oilman and remains steadfast against bringing our troops home? Hemp petroleum is the way to alleviate us from dependency on foreign oil. That is a wonderful dream, cannabis as the means to bring our troops home!

Email replies to the original article mainly asked WHY? Why don't the authorities appreciate the profits to be made from hemp products? Money rules the world, so why don't the industrialists make it legal? Tax hemp, regulate it, make it legal not for altruistic purposes but for their own high profits! The planet and its people would be much healthier. Why the HELL isn't this done?

There are no easy answers. Generally, my reply to these readers scratching their befuddled heads was 'secret societies.' We are not educated on how the world really functions. We grow up watching movies and television where the good guys always win. We are trained to think that our government is good and out for the benefit of all. Films portray the heroes as CIA, FBI, NSA agents or obedient soldiers and police. We are given the false hope that governments care. Please do not be so naïve as to think this is true.

Power was never in the hands of the people. It is almost foolish to think that we, the general masses, control anything. Do you really think petitions work or writing to your congressional representative?

The truth is governments only care about themselves; we are their enemy and they need to control us (because we have the numbers) any way they can...with religion, bogus science, television, news, propaganda, commercials or via the abolition of MARIJUANA.

Instead of THEM industrializing hemp to save the planet...they have banned it. They have created a monster out of the very thing that is a precious gift to the world. NO, it does not make sense at all. However, it is understandable in the insane, Bizarro, backwards world that we live in. Apparently, any good thing is always destroyed. (Ever hear of Nikola Tesla?) Why? The answer must be POWER and CONTROL. The 'haves' do not want to give anything to the 'have-nots.' These are policies from the Dark Ages; they remain alive and well to this day. Research Secret Societies and you might understand.

They invented the monster of MARIJUANA. ('If we do not have an enemy, they will create one'). They had to change the word since the world would never have been turned against HEMP. For thousands of years going back to the wisdom of the Egyptians, hemp was employed for the betterment of the human race. Enter 'marihuana' in the 1930s; just when farm machinery improved that would have made hemp the first 'billion dollar crop!'

Have we grown up enough to see that they have pulled the wool over our eyes? We can no longer afford to be silent lambs. We have to speak up loud and strong and do what we can to spread the truth. What can we do? Share the information, wake people up to the true facts and suppressed knowledge concerning hemp. Eventually, when the pro-hemp people become the large majority, then, the world will change for the better. In the end, we will win.

A very important aspect of those email responses to my original article came in the form of personal stories. These testimonials answer the question: Does hemp help or hurt? Now, this writer could not please everyone on such a controversial subject. Not all the emails contained high praise. There were those anti-hemp fanatics spouting rhetoric from every commercial from the 'Partnership of a Drug-Free America.' Some stated it causes 'lung cancer' as they tried to educate me on the 'toxins' in marijuana.

Well, there are no toxins in hemp. I would love to tell Judge Judy, who is always yelling for someone to take a hair follicle test, the reason why THC stays in your system for so long. (And it's 'hemp' not 'marijuana'). If it were a toxin, a poison or some foreign substance, the body would immediate reject it and expel it. The body does NOT. It knows THC, the active ingredient of hemp, is GOOD. As mentioned before, there are essential fatty acids in hemp seeds. There are other sources in nature for these essential acids, but eating hemp seeds is a great source of (important) proteins. Our own bodies tell us the truth. Shouldn't we listen to the truth within ourselves rather than the garbage broadcasted over television?

Seriously, the pro emails compared to the con emails ran 100 to one. For every one who blasted me, calling me names (these were from mainly young people)...there were about a HUNDRED administrators, educators, some scientists, activists and average people who related their (positive) private stories to me. Many claimed that normal medical procedures and doctors failed them miserably. The ONLY relief from their illnesses was illegal hemp. Here is a peek into some of the best and positive emails over the last two years. They are anonymous and edited, but they are real with the date I received them:

6/20/05 I was diagnosed with an AVM (Arterio Venous Malformation), a neuro-illness that causes me to have fits. On my second appointment

with my neuro surgeon we went through the list of narcotics I was using at the time (marijuana). With that she gave me a sly look and said 'off the record Mr. _____, this will actually reduce your blood pressure and can only be beneficial for your epilepsy. But I didn't tell you that.'

6/23/05 I took a college class in drug use, it was about street drugs, not pharmaceutical drugs. I learned that, at one time, marijuana was legal and was prescribed by MDs and dispensed by pharmacists for a variety of health problems - you know: HTN, insomnia, glaucoma, asthma, etc. I was told that the government decided to impose a $25 tax on MDs who prescribed it and a $35 tax on pharmacists who dispensed it. In protest, both groups elected to stop dealing with it. In response, Pres. Harriman included marijuana in his Narcotics Act of 1914: It was called the Harriman Act. They lumped it in as a class 4 narcotic meaning that there was no medical use for it, even though it's not a narcotic and no one had any indication that it was harmful in any way. In the class, we were shown pictures, taken during the early part of the century, of children smoking a joint...which, we were told, they could purchase for 5 cents, OTC, at any pharmacy, perfectly LEGAL - they were called 'muggles.'

6/18/05 You didn't mention the most important reason why there is a continuing battle against legalization of marijuana, that is because it actually is used to treat sick people with pain successfully and with no serious side effects, something the legal drug industry hasn't accomplished with all their patented expensive crap they call medication. They don't want to loose their chokehold on the billions of dollars they make with this phony medication that could be replaced with an herb anyone can grow in their backyard.

6/18/05 I have been smoking pot since 1962 and I am now 83. I wish I had started when I was a teenager. Good for you.

6/18/05 I have a lot of respect for cannabis as I have used it medically for a spinal injury for several years recently, when all else failed, as doctors gave me narcotic drugs to relieve pain, and these drugs never worked and I was in such severe pain even with their drugs. Cannabis was the only pain reliever that worked. I have done a bit of research myself and found that cannabis is listed under a narcotic and this must be taken off the list that the United States has placed it as narcotic as opium, heroin, cocaine is totally ridiculous and bogus. I feel that if it was taken off the list and proved to the DEA, FDA, or whoever else...that it isn't a narcotic then we can get some headway on this wonderful, helpful plant. If it wasn't for cannabis I wouldn't have healed...The AMA is the worst, the most horrendous of all, because they encourage the doctors to prescribe drugs that lead to the deterioration of the body. Yes, it is a conspiracy, from the beginning, that the corporations don't want hemp to be grown. If they did, the logging industry would be wiped out. I live in southern Oregon and it is the biggest industry here...

6/19/05 I'm a 41 year old victim of Cystic Fibrosis, a genetic disease that kills people usually around 32 years old. I have the worst type of genetic mutation but still alive at the amazement of my Doctor. The reason? Maybe HEMP! I use it in a moderate way, around 2-3 times a week, usually eating it, and the benefit of that medicine is very good for me. Cystic Fibrosis kills people by pneumonia. An effect of hemp is drying mucus secretion so it kills bacteria by starvation. Even better: an antioxidant property of hemp that prevents lung tissue degradation. Another characteristic of Cystic Fibrosis is destruction of the digestive system, especially pancreas, stomach, liver and intestine. That is appetite inhibiting. I'm often eating because of holy hemp. It avoids me from losing too much weight and helps my defense against the constant lung infection. If my doctor knew the truth, he'd surely start hemp harvesting for sure!

6/20/05 I am a Disabled Veteran who spent 17 years taking the VA's Medication. I moved to Arizona; and a Pima Indian Medicine Man gave me an 'herb' to use as a tea to lower my blood pressure. Within 3 weeks it had gone from 210/146 to 130/70. Obviously, I quit the Government Medication and I now participate in the interesting hobby of home gardening of herbal cures for the ills of mankind! By the way, just so you don't think I'm 'a doper,' I served 8 years in the USAF, 2 years as a Palm Beach County FL Police Officer and then 17 years as a Paramedic. I have a Masters of Theology degree from Indiana Wesleyan University. Thanks for having the guts to publish THE TRUTH.

6/23/05 Doug, I also referred Montel Williams to your article about hemp 'marijuana' because he has multiple sclerosis and wants to legalize marijuana for medicinal purpose since it seems to help ms folks with pain. Since he has a TV talk show, I thought maybe he would give your article some publicity.

6/30/05 I am bipolar and when I smoke I am calmer then I am on my pills. I also have fibromyalgia and when in pain I grab my trusty friend Pot; it helps me function and eat. If I didn't have it, I would be dead years ago. Some days I can hardly make it down the stairs because of the ache and pains in my joints. Also, I suffer from severe migraines where I can't even open my eyes because I am light sensitive then my head pounds. I have been on many narcotics from vicodin to oxycottin and they all make me feel worse than smoking a damn joint. Only in America would they make a plant so beneficial to humankind, the most dangerous 'drug' in all the land. If I didn't go score I would be in pain and would never be able to walk out to get my mail. My fiancé gets upset since I can't work but she knows I can't help it. I want to work. I want to be the breadwinner of the family, but physically I cannot maintain standing for more than a few moments before I feel like I am passing out because of the pain. I went to numerous doctors and told them that pot helps me...all of them said since I live in Ohio, they cannot do anything for me besides narcotics...but I cannot smoke a joint...that is rubbish in my book.

7/31/05 I don't know if you have thought about this before, but after all the research I have done, it is my opinion that JFK was assassinated over hemp. He was a known user for his back pain, he fired Anslinger as soon as he got into office and it was said he was going to legalize it in his 2nd term. He never made it to his 2nd term. Maybe you can dig a bit more on your end and see what you come up with to support this theory of mine. It makes sense, don't you think? Keep educating people one day at a time...it's our only shot.

11/18/05 The actual truth that the oil giants do not want anyone to know is that most ‘non producing’ oil fields (for example, Derrick Island off Louisiana) have been found to have mysteriously refilled themselves after closing them down. This is because the vast majority of oil is not biotic but abiotic. The planet’s crust produces billions of new gallons of crude annually and this is refilling the fields pumped out in almost every case. That is why Saudi Arabia just doubled the estimate of its known reserves over its predictions of 10 years ago. The wells keep refilling themselves due to planetary mechanics. Biotic oil is a discredited theory. There was never enough biomass in any prehistoric period to produce the amount of petroleum we have already consumed on this planet to date.

1/7/06 The most important health benefits come from smoking (or otherwise consuming) hemp/cannabis plant material which contains high levels of the active ingredients (THC, etc.) The higher the level of active ingredients, the more medicinal and beneficial it is. This is highly effective medicine, with no negative side effects, which is being denied to people in desperate need because of a pack of lies!

12/10/06 Weed is unprocessed and natural, as opposed to other processed drugs. I have eaten hemp seeds and have even had hemp oil and see the unspoken benefits from them even before I was planning to research the subject.

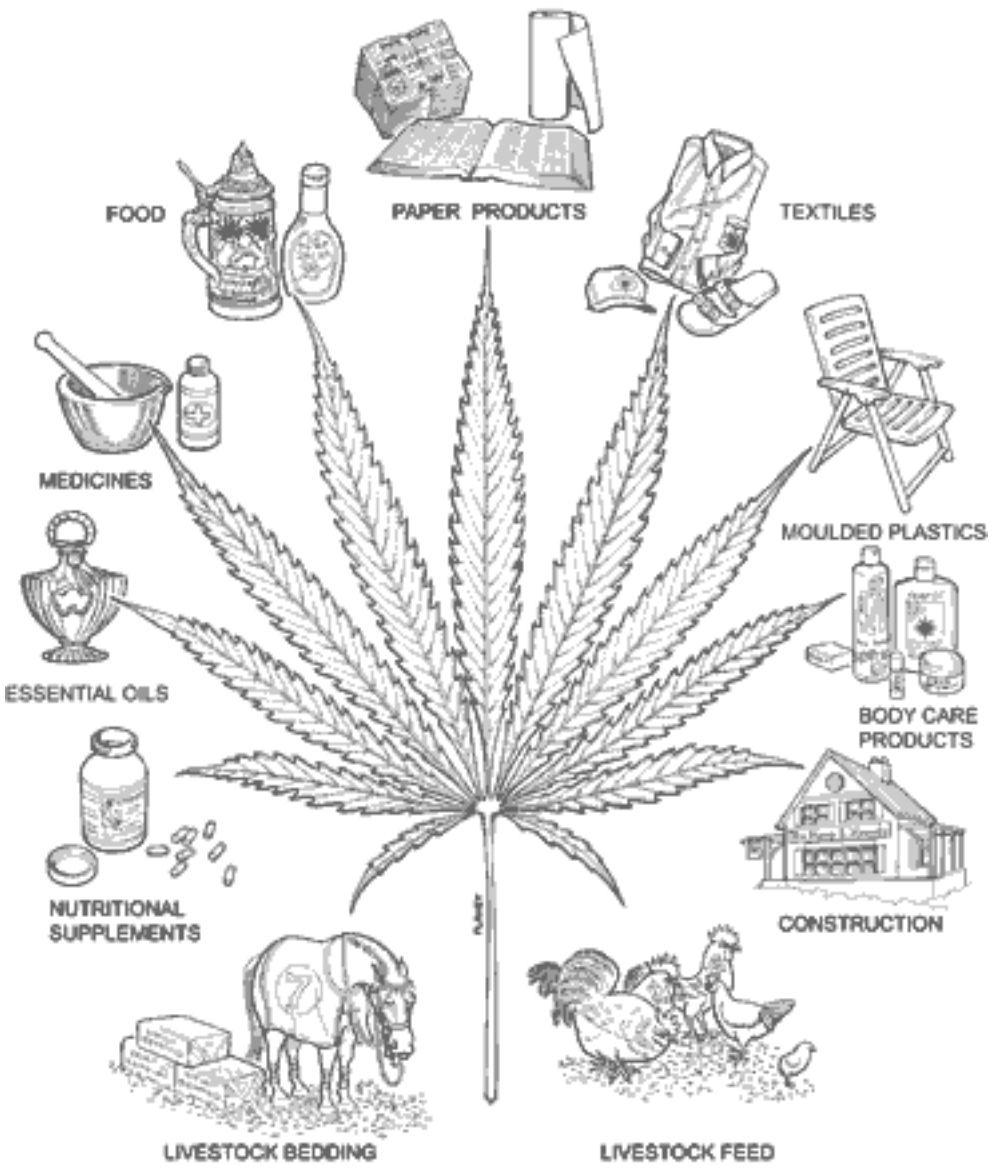
12/10/06 Maybe by the time I retire I can smoke a joint in peace, not fearing the Police and my government. But, other times it just seems hopeless, but I refuse to believe that money is the most powerful force in our Universe...there must be some room for truth.

12/16/06 I am a recreational therapist working for a state operated drug treatment center. If I were to speak in these terms, I doubt I would remain in favor of those above me.

2/2/07 As a medical marijuana user for many years, I would not be here without it. I was a vegetable on the prescribed medication and don’t remember 2 whole years of my life while I was on morphine. Now I live a full and active life, running a business and bringing up a family. Is it too late to change views? I do hope not because it has so much to offer.

Above are some of the best comments from hundreds of replies. My article was far from perfect. I thank those more informed on plants than myself that told me there are other plants that physically reveal their sex such as the holly plant. Many criticized my belief that cannabis had an extraterrestrial origin. That conclusion came out of decades of studying our ancestors of prehistory. My studies have shown a completely new genesis for the human race where we have lost great knowledge. (It may have been advanced HUMANS that created Earthly animals and plants). Cannabis Sativa and all of its infinite and vital uses may be evidence that some great intelligence (with a plan) manufactured it. ‘Chance’ cannot account for its abundant purposes. To some people, the existence of hemp might be proof of the existence of GOD! My website link explores multiple controversies and truths that will never be aired on television. Check it out:
http://www.yurchey.com

Writing about hemp, answering the emails and the entire Internet experience has taught me that there are all kinds of people. You cannot please everyone. What was ‘brilliant’ to one person was contemptible to another. People ‘think’ they know the truth, but the truth is they only know what they have been programmed to think. Very few people do independent research. That’s too much work; they prefer sitting back, watching TV and letting others do their thinking for them. They do not QUESTION what has been handed them. But, if they did explore other possibilities outside of the norm...if they dreamed of a different world...and entertained the idea that we have been lied to by our leaders... maybe we would learn a little and come closer to the FREEDOM that we all rightfully deserve. A cleaner, safer planet will happen. It may not be soon, but it will happen in time...



Dedicated to V for Vendetta and those with the courage to change the world for the better!

dugko@surfside.net

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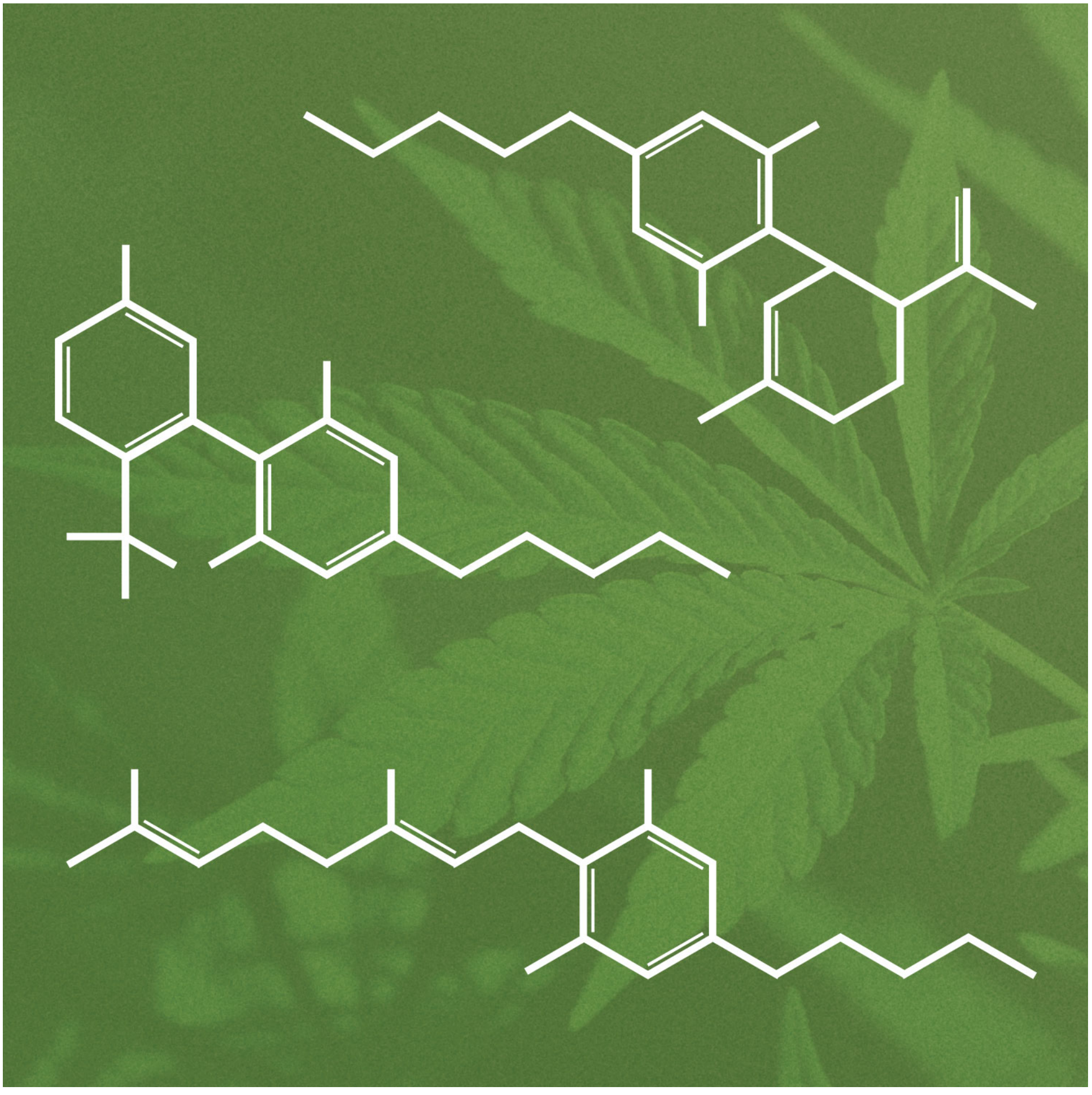


Certain chemicals contained inside of the cannabis plant called cannabinoids might be the most powerful and non inhaled edible nutrients mankind will ever know. They may do fore medicine what germ theory and antibiotics did ushering in the next grate advancement in human health and life expectancy. Cannabis was used as a medicine for thousands of years and was even part of the US pharmacopeia from 1850 to 1942 at witch point it was removed from the American medical establishment and made illegal for all purposes based on races fears and claims that its recreational use gave black men the ability to seduce white woman, that weed caused insanity and schizophrenia, weed gave Mexicans super human straight turning them into blood thirsty violent murders and rapist, that weed led to pore choices such as breaking glass coke bottles open to drink resulting in swallowed glass, that marijuana is deadly resulting in death by smoking it, was a stepping stone leading to hard core drugs such as heroin. Now the new lies are the use and distribution

Of weed propitiates gang wars and terrorism. today cannabis is filed as a schedule one drug under the controlled substances act, a category under which it is legally defined as having absolutely zero point zero zero excepted medical use and a high potential for abuse. This being true despite the fact that eighteen states including Washington DC recognize cannabis as a medicine. The public has known about the incredible medicinal Qualities of cannabis for decades now and has thought to allow it nationally as a medicine. The united states federal government has been extremely firm in its position fighting lawsuit after lawsuit to keep cannabis in its highly restrictive category. The thing is the feds position on the matter explicitly proscribes smoking as the method delivery to the body which they claim to have zero medical value. Generally speaking burning a substance, destroying most of it and inhaling the smoke that's created isn't the most effective way to deliver nutrients to the body. Throughout time the feds have never publicly acknowledged the possibility of eating cannabis cannabinoids as medicine. What's interesting about the stubborn stance government leaders has that cannabis has zero medical value is that they actually hold a US patent on the use of cannabis cannabinoids for the treatment and prevention of ischemic age related inflammatory and autoimmune diseases. The feds have known about the disease fighting exogenous chemical composition of cannabis since 1974 when they discovered the exogenous cannabinoids inside of it to have cancer fighting properties in mice. The national cancer institute of federal

agency tested four common cannabis cannabinoids with lung cancer and on lung cancer cells in test tubes the cannabinoids were administered orally and ingested by the mice three out of the four cannabinoids reduced the size of some tumors and slowed the progression of others leading to a longer survival time than the untreated mice. The test tube cancer cells were also impaired by the cannabinoids. At that time science had no explanation why cannabinoids could show to be anti cancerest. The pieces started to come together in 1988 when endogenous cannabinoid receptors or endocannabinoid receptors was found in the brain of a rat soon after it was discovered that endocannabinoid receptors are replete throughout the mammalian body. They are in every bodily system, brain, blood cells, internal organs, skin, and bones. These receptors are not unique only to mammals. Every living thing that has a back bone and a central nerves system has endocannabinoids receptors. In mammals these receptors have been found to play a kea role in many bodily functions. Two distinct types of cannabinoid receptors have been identified and a third type is expected to exist. the two distinct types of endocannabinoid receptors help control different parts of the body in different ways. the endocannabinoid receptors are in charge of turning up activity in certain parts of the body and turning it down in others. Acting as regulators to keep bodily functions in check. Then in 1992 it was discovered that the body actually makes its own form of cannabinoids called endocannabinoids to feed there receptors and to allow them to do there important over cite job eating foods with omega 3 fatty acids helps the production of endocannabinoids such as anandamide or 2-erekidonoyalglycerol 2-AG which are thin created on demand by the body to feed any receptor that needs stimulation. The endocannabinoid 2- arachidonoylglycerol (2-AG) is in mothers milk. Scents discovering that endocannabinoid receptors impact apon so many bodily functions, scientists have been determined to experiment with chemicals that effect them and explore for their potential medical use. There have only been five distinct types of endogenous endocannabinoids identified to have been made inside the body there have been 85 uneak exogenous exocannabinoids identified so far in cannabis. There are cannabinoids in some other plants like Echinacea, but no where near the quantity or potential medical quality of cannabis. Because the 85 known cannabis cannabinoids are illegal to proscribe and prohibitively difficult to research for medical use in America researchers instead have been forced to create artificial cannabis mocking drugs to attempt to stimulate the endocannabinoid receptors and manipulate their actions in a helpful way. Schematic representation of the endocannabinoid system at a synaptic cleft. Endogenous cannabinoids are synthesized from membrane lipids in postsynaptic neurons after calcium (Ca++) increase . They diffuse and activate pre-synaptic CB1 receptors that indicate intercellular cascades, inhibiting neural activity and neurotransmitter release. Anandamide (arachidonoylethanolamide, AEA) undergoes re-uptake and hydrolysis by fatty acid amide hydrolase (FAAH) whereas 2-arachidonoyl glycerol (2AG) is broken down by monoacylglycerol lipase (MAGL). The existence of a membrane endocannabinoid-transporter (T) is controversial. The technical definition of a cannabinoid is any chemical that is capable of binding to and activating an endocannabinoid receptor. Cannabinoids made inside the body are called Endo (5+) endocannabinoids cannabinoids found in plants are called phyto(110+) cannabinoids and the rest, Artificial (17+) are made by scientists in a laboratory. These man made cannabinoids don't suffer the same automatic illegal statist as their natural counterparts. Section 812. Schedules of Controlled Substances (a) Establishment. There are established five schedules of Controlled substances, to be known as schedules 1, 2, 3, 4, and 5. Such schedules shall initially consist of the substance listed in this section. The schedules established by this section shell be updated and republished on a semiannual basis during the two-year pried beginning one year after October 27, 1970, and shall be updated and republished on an annual basis thereafter. (b) Placement on schedules; findings required Except where control is required by United States obligations under an international treaty, convention or protocol, in effect on October 27, 1970, and expect in the case of an immediate precursor, a drug or other substance may not be placed in any schedule unless the findings required for such schedules are made with respect to such drug or other substance. The findings required for each of the schedules are as follows

(1) Schedule

1.– (A) the drug or other substance has a high potential for abuse.

(B) the drug or other substance has no currently accepted medical use in treatment in the United States.

(C) There is a lack of accepted safety for use of the drug or other substance under medical supervision.

Synthetic cannabinoids that gain FDA approval are allowed to be marketed and soled on the highly expensive pharmaceutical market. Today there are two such artificial cannabinoids that can legally be prescribed to Americans. In 1985 the drug merijuanal was approved by the FDA and then entered the market. It is proscribed to reduce nausea and vomiting in chemotherapy patients and increase appetite in aids and chemo patients. Also in 1985 the drug Cesamet was approved by the FDA but it was not allowed to enter the market until 2006 it is proscribed to reduce nausea and vomiting and neuropathic pain in chemo patients and increase appetite in aids patients. Both merijuanal and Cesamet are modeled after the cannabis cannabinoid delta-9-tetrahydrocannabinol THC the only other pharmaceutical cannabinoid drug in the world is the low concentration oral spray Sativex. It interred the market in 2003 as a treatment for multiple sclerosis patents to alleviate their spasticity and neuropathic pain. Because Sativex contains the real cannabis cannabinoids THC and CBD it is illegal in America but available in other countries like Candia, England, Spain, and New Zealand. Although merijuanal and Cesamet are the extent to which an American can legally used cannabinoids the research shows endocannabinoid receptors could be used to do more than just fight symptoms. A search on PubMed.gov for the word cannabinoid yields 15243 result. These studies most of which involved non smoked cannabinoids show that endocannabinoid receptors

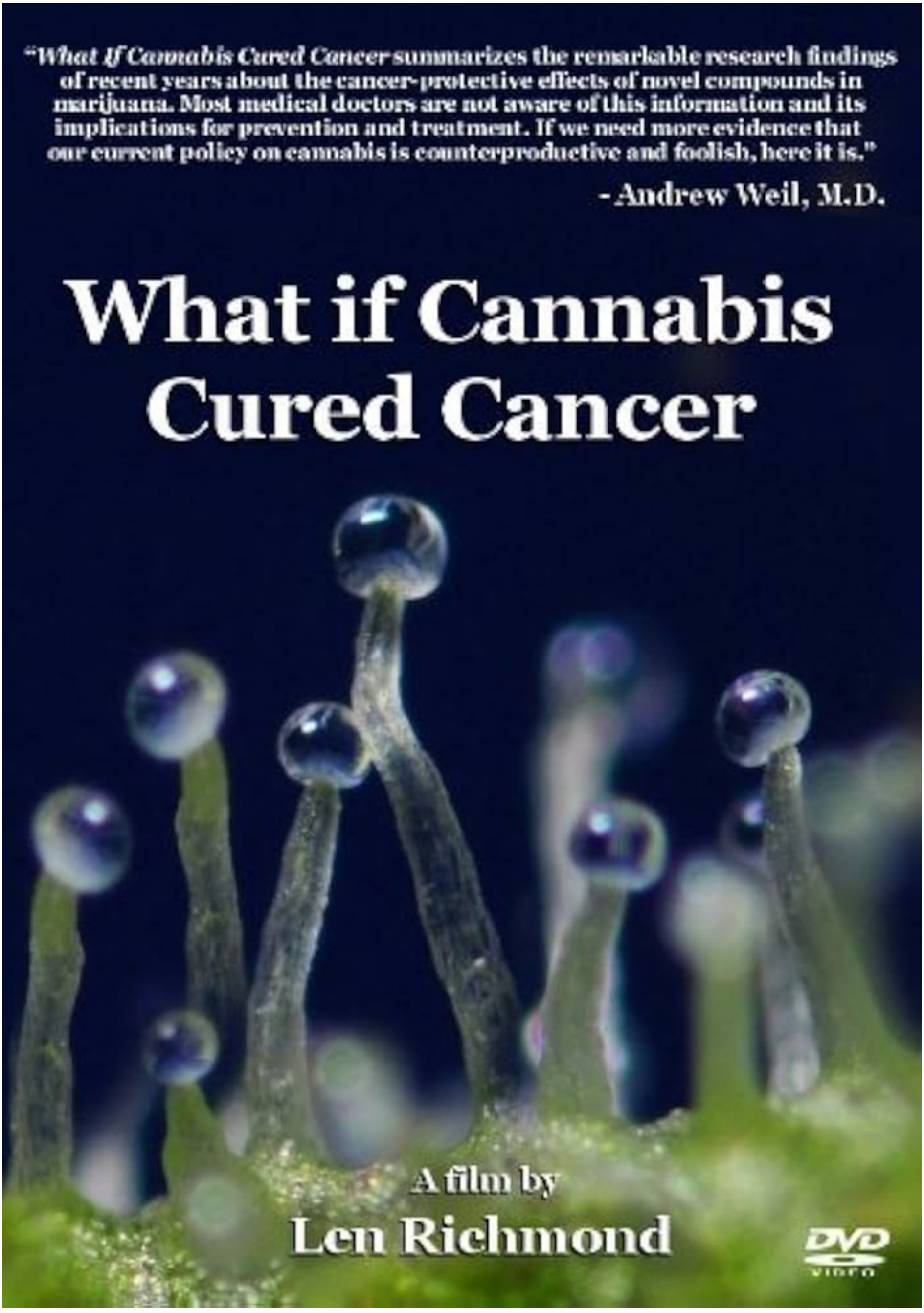
Could be utilized to have a uniquely profound impact on what is currently known as health. Schematic representation of endocannabinoids action. Endocannabinoids are synthesised in and released from the membrane of post-synaptic neurons on-demand after calcium influx (1). They activate pre-synaptic CB1 receptors and restrain neural activity

(2) Anandamide and 2- arachidonoylethanolamide (2AG) are removed from the synaptic cleft by up-take into the post- and pre-synapse, respectively. (3). Once inside neurons, anandamide binds TRPV1 (with consequences opposite to those of CB1 activation) and undergoes hydrolysis by FAAH, whereas 2-arachidonoylethanolamide, (2AG) is hydrolysed by MGL

This is no where near a complete list but here are 25 studies showing different diseases and conditions that could be treated or even healed by

exploiting the endocannabinoid system
breast Cancer artificial cannabinoid used in order to reduce the size and spreading of tumors
"Mice treated with JWH-133 or WIN -55,212-2 (artificial cannabinoids) showed a 40% to 50% reduction in tumor growth and a 65% to 80% reduction in lung metastasis."
Prostate Cancer an artificial cannabinoid reduced tumor growth
"WIN-55,212-2 (mixed CB1/CB2 agonist) (artificial cannabinoid) treatment...resulted in a dose- and time dependent inhibition of cell growth."
Lung Cancer artificial cannabinoids stop the growth and spreading of tumors
"Both CB1 and CB2 agonists Win55,212-2 and JWH-133, respectively, (artificial cannabinoid) significantly inhibited in vivo tumor growth and lung metastasis (50%)"
Pancreas Cancer artificial cannabinoid inhibited tumor growth
"(artificial cannabinoid) treatment strongly inhibits growth of human pancreatic tumor cells xenografted in nude mice without appearing toxic effects."
Liver Cancer THC and an artificial cannabinoid impaired cancer cells
"We found that THC and JWH-015 (an artificial cannabinoid) reduced the viability of human hepatocellular liver carcinoma cell line and hepatocellular carcinoma cells"
Cervical Cancer an artificial cannabinoid killed cancer cells
"MA- (artificial cannabinoid) induced apoptosis was confirmed in another human cervical cancer cell line"
Colon Cancer an artificial cannabinoid reduced cell growth and killed cancer cells
"Rimonabant (an artificial cannabinoid) significantly reduced cell growth and induced cell death"
Non-Hodgkin Lymphoma an artificial cannabinoid reduced tumor size
"In vivo treatment with R(+)-MA (an artificial cannabinoid) caused a significant reduction of tumor size"
Leukemia Delta-9-Tetrahydrocannabinol THC reduced tumor size and killed cancer cells
"Treatment of EL-4 tumor-bearing mice with Delta-9 tetrahydrocannabinol THC in vivo led to a significant reduction in tumor load, increase in tumor-cell apoptosis, and increase in survival of tumor-bearing mice"
skin cancer artificial cannabinoid reduced the growth of tumors
"Local administration of the mixed CB(1)/CB(2) agonist WIN-55,212-2 or the selective CB(2) agonist JWH-133 (artificial cannabinoid) induced a considerable growth inhibition of malignant tumors"
Cirrhosis an artificial cannabinoid reduced fibrosis
"fibrosis was significantly reduced in rats treated with Rimonabant (an artificial cannabinoid) compared with rats receiving the vehicle."
Hepatitis Delta-9-Tetrahydrocannabinol THC inhibited hepatitis
"Intraperitoneal administration of THC... inhibited hepatitis as shown by significant decrease in liver enzymes and reduced liver tissue injury."
Alzheimer's an artificial cannabinoid protected neurons and reduced cognitive impairment
"CB1 Agonist ACEA (an artificial cannabinoid) protects Neurons and Reduces the cognitive impairment of...mice."
Parkinson's THCV could slow progression of PD
"THCV has a promising pharmaceutical profile for delaying disease progression in PD"
Multiple Sclerosis artificial cannabinoid reduced neurological disability and progression of the disease
"the treatment of mice experimental brain inflammation with the cannabinoid agonist WIN55,12-2 (an artificial cannabinoid) reduced their neurological disability and the progression of the disease."
Arthritis artificial cannabinoids protected cartilage from damage and degradation
"It is concluded that biologically stable synthetic cannabinoid protect cartilage matrix from degradation induced by cytokines"
Cardiac Arrhythmia Anandamide and an artificial cannabinoid prevented arrhythmias
"It has been found that prior intravenous administration of the endocannabinoid anandamide (10 mg/kg) or it's synthetic analogue R-(+)-methanadamide (5mg/kg) prevents a development of ischemic and reperfusion arrhymias in rats
Insomnia an endocannabinoid increased sleep
"Anandamide (an endocannabinoid) increased adenosine levels in the basal forebrain and also increased sleep."
Sleep Apnea cannabinoids suppressed sleep related apnea
"This study demonstrated potent suppression of sleep-related apnea by both exogenous and endogenous cannabinoid."
Inflammatory bowel Disease artificial cannabinoids reduced inflammation
"Inflammation was significantly reduced in the presence of UBR597, VDM11, or both (artificial cannabinoids)"
Depression Endocannabinoids may treat depression and anxiety
"Animal experiments suggest that drugs promoting endocannabinoid action may represent a novel strategy for the treatment of depression and anxiety disorders."
Post Traumatic Stress disorder an artificial cannabinoid could help control nightmares
"The results of this study indicate the potential benefits of abialone, a synthetic cannabinoid, in patients with PTSD experiencing poor control of nightmares with standard pharmacotherapy."
HIV cannabinoids inhibited TAT
"Cannabinoids inhibit migration of microglial-like cells to the HIV protein TAT."
Osteoporosis an artificial cannabinoid increased bone mass and protected against bone loss
"Pharmacological and genetic inactivation of CB1,

CB2 and e GPR55 in adult mice suppress bone resorption, increase bone loss"
Nicotine Addiction an artificial cannabinoid could help smokers quit smoking
"These findings indicate significant, but limited, utility of e rimonabant (an artificial cannabinoid) for smoking cessation."
The bulk of trials that do moved on to humans are those that show potential that control symptoms. For some reason almost no experiments that show potential to actually cure disease move on to humans interestingly enough in all of history there hasn't been one study done testing the curative effect of ingesting cannabis cannabinoids on humans. The only study on humans testing the disease fighting properties of non smoked cannabis cannabinoids took place in Spain in 2006 they took the lone cannabis cannabinoid chemical THC and directly injected it into malignant brain tumors of patients. As suspected it had a cancer fighting reaction in the patients tumors which allowed them to live much longer than expected. Since then no more clinical trials of this nature have been preformed on humans. Despite the mountains of research showing the disease fighting exogenous plant based chemical cannabinoid composition potential of cannabis, both artificial cannabinoids available to sick Americans are modeled after the cannabis chemical THC and all they do is alleviate a few symptoms in 2007 using a new method to target the endocannabinoid receptors a French pharmaceutical company Sanofi Aventis who's logo is because health matters, came close to bringing America its next cannabinoid drug. They theorized that because CB1 receptors stimulate appetite, shutting those receptors down could help reduce appetite and help people lose weight. They didn't know exactly which CB1 receptors controlled appetite, so they made a drug that simple blocked as many CB1 receptors as possible. Their drug called Acomplia made it all the way to a FDA faze three trial patient were indeed showing signs of reduced appetite. The drug also showed potential uses for nicotine Addiction and metabolic disorders but the FDA stopped the trial. Some patients formed extreme anxiety and depression and attempted suicide. Patients with a history of depression weren't allowed in the study although Acomplia was not approved in the US it was approved in Europe and it was available in 56 countries by 2008. it crinkly became clear however that its negative effects out wade it's positive effects and it was polled from the market by the end of 2008. other pharmaceutical companies like American Pfizer were in reproduction stages with their own versions of weight loss CB1 receptor blockers. These programs were not fully carried out because of their failure of Acomplia. What Acomplia did is show clearly that if at lease certain CB1 receptors did not receive endocannabinoids then the body could not function normally. Plain and simple cannabinoid receptors are part of the body and they need to be fed. In todays world do to one or a combination of certain factors such as age, weight, diet, lack of diet, free radicals, and environmental
Toxins, fore some people there could be a problem some where in the line of producing, transporting, and receiving all of the known endocannabinoids needed to keep their Endocannabinoid System receptors happy. Thus directly impacting proper function in specific parts of the body. A lack of Vitamin A leads to Exophthalmia-night blindness, a lack of Vitamin B12 leads to Megaloblastic Anemia-Subacute Combined Degeneration of Spinal Cord, a lack of Vitamin C leads to Scurvy, a lack of Vitamin D leads to Rickets, a lack of Vitamin K leads to Coagulation, a lack of Thiamine leads to Beriberi, a lack of Niacin leads to Pellagra, a lack of Endocannabinoids leads to depression. Over time this could lead to a condition similar to avitaminosis. Avitaminosis is any disease caused by a chronic long term prolonged Vitamin deficiency its already known that suppurating serten CB1 receptors for a short time increases the chances of depression. Considering the multitudes of bodily functions the CB1 and CB2 receptors control as a whole, who knows how many other conditions could be caused by a lack of nourishment in the receptors. Vitamins were only discovered because of the realization that if people eat certain plants they didn't get serten disease. The cure for the disease was as simple as eating those plants containing nutrients nature intended for the body. And as long as people got those nutrients regularly then disease would never develop. The chemicals in cannabis meaning both hemp and marijuana when they are fresh raw and unheated are not psychoactive. THCV contained only in living cannabis can't get you high. That means as long as you eat or juice it in its natural state cannabis is just another vegetable most modern pharmaceutical drugs focus only on a small concentrated part of the body and work to shut it down. it's possible that when ingested properly at least one of the 85 natural cannabis cannabinoid could safely fill the role of the endocannabinoids around the entire body taping into a natural built in system to help restore normal order to arias of the body that need it in a way that has never been intentionally utilized in all of known human history. The government will not let cannabinoids be officially fully tested as in the prevention or cure of disease, thus not allowing scientists to know the full potential of cannabinoid therapy in the roll as medicine or its nutritional value in the health and betterment of human kind. their are a lot of people out there who are claiming that ingested cannabis cannabinoids have cured their illnesses but their accounts are only anecdotal. Rather ingesting Cannabis cannabinoids fights deficiency in the body, provides an incredible boost to the health, or dose nothing at all we deserve to know. If you care about the millions of sick, the injustice, the out of control cost of health, care and the underfunded medicaid programs then position against the prohibition of natural therapies which are all being made illegal. The only way the government will ever do anything is if enough people speak out. The world can't afford to ignore cannabis any longer.


"What If Cannabis Cured Cancer summarizes the remarkable research findings of recent years about the cancer-protective effects of novel compounds in marijuana. Most medical doctors are not aware of this information and its implications for prevention and treatment. If we need more evidence that our current policy on cannabis is counterproductive and foolish, here it is."
- Andrew Weil, M.D.
What if Cannabis Cured Cancer
A film by
Len Richmond
DVD
VIDEO

What if Cannabis Cured Cancer

It's been used by human bings since the dawn of time. Early mans diet undoubtedly included cannabis since it grew Prolifically in Africa and Asia. Pot is highly nourishing due to its protein, fiber, 8 amino fatty acid, omega 3 content. Cannabis is speculated to be one of the first

cultivated plants. In resent years the hemp plant has been proven to be anti-spasmodic, anti-inflammatory and anti-bacterial. It can treat depression, dramatic stress syndrome, chronic pain, glaucoma, migraines, multiple sclerosis, turrets, nausea, cerebral palsy, epilepsy, cancer, and a host of other elements without the unwanted side effects of pharmaceutical drugs. Historical evidence showed that the holy anointed oil used by old testament Hebrews contained cannabis extract. The early Christians used cannabis as part of there baptismal process. In the eighteen hundreds medical journals published more than a hundred articles on the therapeutic use of the drug then known as cannabis indica or indene hemp. Cannabis was often prescribed by doctors and was part of every medical bag right through the early part of the twentieth century. It was a popular treatment from labor pains, asthma eremitism and nerves disorders. It was even given to cranky babies to get them to stop crying. Then the dark days for pot began in the early part of the twentieth century with an advent of chemically created drugs and an emerging profit driven assembly line medical system. The NEW ORDER didn't want competition. Cannabis was still preferred by many patients and doctors to the new miracle drugs which were composed mainly of unnatural toxic chemicals. After lobbing by the pharmaceutical industry and fear mongering misinformation by there donation dependent allies in congress, a federal law passed in nineteen thirty seven that prohibited doctors from proscribing pot in any circumstances with threat of prosecution. Even though it was strongly apposed by the American medical association of its day, all cannabis products were removed from the us pharmacal burial national formulary by nineteen forty two. After alcohol was relegalized, rather than go out of business the massive bureaucracy of prohibition turned its operation to hemp eradication. This war on cannabis lasted until just a few years ago when states started passing their own medical cannabis initiatives in order to once again provide pot safely to patients. California was the first U.S. state to pass a medical marijuana law in nineteen ninety-six. The intent was to give relief to patients suffering from chronic pain, aides cancer and other illnesses. Sense nineteen ninety-six thirteen other American states have passed similar compassionate use laws, twelve more had ballet measures or legislation pending. Although cannabis smoke has been shown to have precancerous effect in animal tissue, countless study's have felled to find a link between cannabis smoking and cancer. In fact heavy cannabis users were shown to have lower cancer risks than the general population. Is it possible that the tar in smoked pot could be causing malignancies while the chemical constituents in pot are curing them at the same time? Although there's some concern that cannabis can exasperate schizophrenia and psychosis in a few patients already suffering from these diseases resent extensive studies in the U.K. have failed to hold support a causable relationship between cannabis and mental disorders. It was only in the 1960's that fat salable THC was isolated from pot plants and declared the substance that got us high. Cannabis can effect memory and movement but only temporarily. Though there have been reports of pots negative effects on the hart and cardiovascular system, they are ware and not scientifically substantiated. Research has also shown that pass claims of brain damage from pot failed to hold up under scientific scrutiny. All in all cannabis is vary low risk. The long history of the human use of cannabis also attest to its safety. Nearly five thousand years without a single documented death. Medical cannabis dispensaries across America are now offering an oppressive array of cannabis enhanced foods which makes it easier to over medicate with cannabis edibles. they are neither toxic nor lethal and the symptoms will were off in a few hours. For cannabis to prove fatal you'll have to smoke fifteen hundred pounds within fifteen minutes and any resulting death would likely be from asphyxiation. Doosan's of major studies have been passed in the last few years that indicate that chemicals in cannabis in the lab and in animals have a significant effect in fighting almost all major cancers including brain, breast, prostate, lung, thyroid, colon, skin, pituitary, melanoma, and leukemia cancers. They do this by promoting the death of cancer cells that have forgotten how to die as well as a reduction in there crucial blood supply while leaving healthy cells untouched. But why you may wonder how would cannabis have any effect on cancer. The answer can be explained in one word. Endocannabinoids. Amazing as it sounds were all born with a form of cannabis already in our bodies. It's called the endocannabinoid system. The endocannabinoid system or ECS influences multi physiological processes. This intercut system moderates energy intake as well as nutrient transport, metabolism and storage. A completely natural collection of compounds, endocannabinoids are our bodies form of cannabis and are involved in most of our cells and structures. They control a variety of functions in the nerves system, heart, reproductive and immune systems. Endocannabinoid messengers help the cells communicate. Typically they protect our good cells while killing the bad ones like cancer cells. In all animals the nerves system is made of the same components. Large numbers of nerve cells carrying electrical signals and where ever the cells meet these signals are passed to a receptor in the next cell by a chemical messenger called a neural transmitter. Inside the brain there are different types of neural transmitters including dopamine and serotonin. All life with a nerves system is integrated with this basic structure. Because of these similarities these new signals came to be known as cannabinoids. It was inevitable that cannabis would eventually meet its perfect partner. Humanity. Rather you like it or not each and every one of us is fundamentally wired to respond to cannabis. After scientist discovered THC, they found that it could bind to specific receptors in the brain and the area around it and that this interaction created a cascade of biological processes leading to the well known high. If you block the endocannabinoids artificially in mice they tend to grow tumors and they also get depressed. A few years ago some brilliant entrepreneurs came up with the idea that blocking the endocannabinoids in our body to create a new diet drug. The theory being if cannabis gives people the munchies, then blocking the endocannabinoids would make them lose their apatite's. The drug they developed was Rimonabant which did indeed decrease apatite by blocking the endocannabinoid receptors, but data from clinical trials showed that Rimonabant users suffered depression, anxiety, insomnia and digressive impulses at trice the rate of subjects given a placebo. In one study there were five suicides among Rimonabant users because as they discovered, endocannabinoids are also mood regulators with the capacity to make us feel euphoric or when blocked depressed. Rimonabant was finally withdrawn from the market in two thousand eight. Researchers at the MD Anderson cancer center in Texas reported that mice on Rimonabant developed Particularly cancerous polyps at a far higher rate than controls confirming that endocannabinoids are not only mood regulators but tumor regulators as well. What's especially fascinating is that while significant numbers of endocannabinoids are not found in breast and prostate cells, the homes for the two leading causes of cancer it appears that large quantities show up only after normal cells begin to progress toward a state of uncontrolled growth in a desperate attempted to restrain the tumorest invaders. The endocannabinoids already inside our bodies did an efficient job for thousands of years working as tumor regulators, free radical scavengers and anti-inflammatory agents. Then once we entered the industrial age with all it's pollution and chemical indoctrine of food, the endocannabinoids became overwhelmed with the task of

keeping us healthy and protected from toxins. Better public health helped us live longer but unfortunately human beings became innovated with the free radicals our bodies produced naturally by simply being alive along with an unprecedented Tyrannical valiance in-doctrine of GMO's carcinogens and contaminates. Including pharmaceutical drugs. The endocannabinoids needed help. ENTERED CANNABIS! When cannabis is consumed and broken down naturally into a collection of cannabinoid molecules the THC and its sisters seek out and bind with the endocannabinoid receptors already inside us. Together they give the body more ammunition agents all of the diseases of modern times. Scientist are now experimenting to see witch parts of the pot plant including its cannabinoids and other biological compounds might be the most effective agents a variety of diseases including diabetes and cancer. Despite all the difficulties there's a group of doctors in the U.S. and abroad who have been advancing the research on the medical use of cannabis with highlly impressive and sometimes revolutionary discoveries. Seth research laboratories in California recently demonstrated that in some tumors cancer cells are killed by cannabis while the other healthy cells are left untouched cells that stop moving and become still white dots are dead cancer cells. The ability of cannabinoids to kill bad cells while protecting healthy ones is Particularly important when we are talking about brain cancer because of the blood brain barrier. The brain has to be sheltered from outside influences that might hitch a ride in the blood stream and cause havoc. What is exiting and unique about cannabinoids is that they can pass through the blood brain barrier because of their slippery fat loving nature. Cannabinoids get right into the brain's cancer cells by moving easily through the cells midbrain which are also composed of lipids. The evidence is piling up in mice infested labs at the endocannabinoid system when stimulated by cannabinoids has an anti tumor effect and can instruct cancer cells to commit suicide. Doctor Seminaire Manuel Guzman is a professor of biochemistry and molecular biology in Madrid Spain and is known for his ground breaking studies on medical cannabis. If cannabis might be the miracle cancer cure everybody's been searching for then why don't doctors everywhere know about it. Why aren’t billions of funds being directed towered cannabinoid research by the organization that raise money for cancer therapies. It's only an aromatic herb yet almost every government in the world restricts or bands Its use. In many countries cannabis suppliers and users can still be put in jail for life or even executed. Why dose the corporate media continue to connect marijuana only with gangs, Violent Drug dealers, race, societies losers and not the growing number of doctors and scientist who are discovering its benefits. It is true that for those who use it regularly pot can change your way of thinking and can make people question why they should continue to do things that aren’t making them happy. In essence grass can make you feel that the past and the future are not as important as the present. It has the potential to altar the vary nature of consciousness by opening up the mind to unpredictable possibilities. That doesn't sit well with those who insists on remaining in control. Cannabis is a natural substance therefor couldn't be patented until the twentieth century. Anyone with a green thumb can grow it. So its production could be in the hands of the people instead of the corporations that run a two hundred billion dollar a year cancer business. It may not be a coincidence that pharmaceutical companies are silently preparing for what could easily become a total revolution in the curing habits of the worlds population. G.W. pharmaceuticals a company that specializes in the study and commercialization of cannabinoids has already established a full cycle facility in undisclosed location in the south of England where they oversee the cultivation of the plant right to the finished medical product. This facility grows thirty to sixty thousand plants every year once the cannabis is ready for processing it undergoes the same exact treatment, extraction and refinement that is forbidden to regular citizens around the world. Cannabis contains four hundred and twenty-one chemicals. 85 chemical compounds from cannabis are cannabinoids. Cannabinoids are a unique class of compounds not found anywhere else in nature. It's a colorful plant with antioxidant activity. Cannabis is subject to few predators and has even been utilized as an insect repellent. Another stress to which pot plants are subject results from their daily exposure to the radiation produced by ultraviolet sunlight. This has created certain Unique resistant defenses in cannabis to ultraviolet U.V U.V.A. U.V.P. solar radiation. The same chemical that protects the plant may well have a preventive anti- cancer effect inside all animal and human cellular reparative structures. Since cannabinoids are scavengers of disease causing free radicals. There are four resistant compounds that are of the most interesting to scientists doing medical research. Delta-9 tetrahydrocannabinol or THC is the principal psychoactive compounded ingredient that creates euphoria when smoked, vaporized or ingested once after it has deride out. THC exerts a wide spectrum of biological effects by mimicking the bodies own natural endocannabinoids. Faced with this rabidly changing scenario the drug enforcement administration is trying to modify it's official position to allow pharmaceutical companies to market THC without appearing to contradict all the tobacco, alcohol, pharmaceutical drug war cabal funded negative lies propaganda information on the Mexican slang word [MARIJUANA] they have been propagating for decades. The DEA now recognizes the medical qualities of THC by trying to shift the blame from the prohibition of hemp to the possible damage caused by the smoke. Cannabidiol, also known as CBD is a non psychoactive component of cannabis that gives the sedative effect to the high experienced amongst its numerous functions. CBD has been shown to have anti-inflammatory analgesic effects. It helps fight diabetes, bacterial infections, and malignant tumors. This compound is also a nerve protectant with well documented anti-psychotic and anxiety reducing effects. Tetrahydrocannabivarin, or THCV has been found in significant amounts mostly in south African and some Thai strains of grass. High concentrations of THCV will make the high come on quicker but last for less time. This chemical compound may eventually prove to be a useful treatment for type two diabetes. THCV has been shown to have a protective, preventative effect against malignant tumors. Cannabichromene, or CBC is a low level cannabinoid though little research has been done scientist believe CBC may have an antidepressant effect as well as the ability to reduce inflammation. CBC strongly inhibits tumor growth in leukemia and breast cancer. Faced with all these new discoveries, one is brought to question what stops cannabis from being legalized for medical use all around the world It's not only doctors calling for legalization. After becoming a perfect fixer in the urban panorama in California Oregon is beginning to open the first public venues for medical cannabis smokers. At the federal level they are still clinging to a more archaic view and equate arkaim with cannabis To discourage church goers from using it under any circumstances. The anticancer qualities of cannabis appear to have been known to the US government for over fifty years. In a nineteen seventy four seventy five medical collage of Virginia study, led by doctor Albert Monson discovered that THC slowed the growth of three kinds of cancer, Lung, breast and virus induced leukemia. The Virginia study was shout down and all further cannabis tumor research was abandoned What is a weed? A plant whose virtues have not been discovered. Ralph Waldo Emerson.

HEMPSECTOR
Natures Cure
Ca N Na B I S

Recommendation	Type	Evidence Quality	Strength
1.1. Health systems and clinicians, in partnership, should provide adults with cancer unbiased, evidence-based cannabis and/or cannabinoid educational resources to facilitate clinical communication, informed decision-making, and systematized approaches to care.	**Good Practice Statement**		
1.2. Given the high prevalence of cannabis and/or cannabinoid use among adults with cancer, clinicians should routinely and non-judgmentally inquire about cannabis use (or consideration of use), and either guide care or direct adults with cancer to appropriate resources.	**Good Practice Statement**		

Note. Clinicians should remain sensitive to cannabis regulations' disproportionate impacts on marginalized communities and work to omit cannabis-related and other biases (eg, racial, ethnic, and socioeconomic) from clinical discussions about cannabis and/or cannabinoids. Table 1 offers suggestions for cannabinoid history-taking.

1.3. When adults with cancer use cannabis and/or cannabinoids outside of evidence-based indications or clinician recommendations, clinicians should explore goals, educate, and seek to minimize harm.	**Good Practice Statement**		
2.1. Clinicians should recommend against use of cannabis and/or cannabinoids to augment cancer-directed treatment unless in the context of a clinical trial.	**EB**	**VL**	**W**
2.2. Clinicians should recommend against use of cannabis and/or cannabinoids in place of cancer-directed treatment.	**IC**	**VL**	**S**

Note. Cannabis and/or cannabinoids used as cancer-directed treatment may cause significant clinical (eg, fatigue, confusion, feeling "high") and financial toxicities without good quality evidence of clinical benefit.

3.1. Adults with cancer who receive moderately or highly emetogenic antineoplastic agents with guideline-concordant antiemetic rophylaxis and experience refractory nausea or vomiting may augment their antiemetic regimen with dronabinol, nabilone, or a quality-controlled oral 1:1 THC:CBD extract.	**EB**	**M** for dronabinol and nabilone **L** for 1:1 THC:CBD extract	**W**
3.2. Outside of a clinical trial, clinicians should not recommend that adults with cancer use 300 mg or more per day of oral CBD to manage symptom burden due to lack of proven efficacy and risk for reversible liver enzyme abnormalities.	**EB**	**L**	**W**

Note. In adult and pediatric populations without cancer, reversible liver enzyme abnormalities primarily occurred in study participants taking 300 mg or more per day of oral CBD.

3.3. Evidence remains insufficient to recommend for or against cannabis and/or cannabinoids in managing cancer treatment-related toxicities or symptoms (including cancer pain), aside from clinical settings addressed in Recommendations 3.1 and 3.2 or within the context of a clinical trial (Table 2 in the full guideline).	**NR**	**Ins**	**NA**

Anonymous Cannabis Cures Cancer
A 60 gram supply of cannabis oil is recommended for those suffering from serious disease such as cancer.
Each tube is 10 grams.
pictured here is enough to treat one cancer patient & only $600 to 1,200 total.
The health care industry would charge you that much for the first night in the hospital.
The United States Government manipulated by the people to hide the federal name, has known since 1974 that cannabis kills cancer.
In 1972 Richard Nixon wanted a larger budget for his war on drugs.
He thought if he could prove that cannabis damages the immune system causing lung cancer like cigarettes do, he would get the support he needed. He gave the Medical College of Virginia, who had been funded by the national institute of health 2 years to do the study on the

effects of THC on the body.
In 1974 the study was completed, it turns out they found instead when THC is ingested in highly concentrated forms, such as eating cannabis oil will attack any mutated cells in your body, why'll strengthening & rejuvenating the healthy cells.
The researchers found instead that THC slowed the growth of three kinds of cancer in mice.
they found the perfect cure for cancer.
It worked fast. It worked well, it worked on many forms of cancer in all stages & it had zero harmful side effects unlike chemotherapy which deteriorates the entire body which is known to kill 1 in 5 patients.
Not only can cannabis cure cancer, it also has been proven that it can dissolve all forms of tumors & can even combat super bugs like MRSA.
When Richard Nixon saw the results of the study, he got furious.
He through the entire report in the trash & deemed the study classified.
In 1976 president Gerald ford had the DEA quickly shut down the Virginia study and all further public cannabis tumor research and granted exclusive research rights to major pharmaceutical companies who set out unsuccessfully to develop synthetic forms of THC that would deliver all the medical benefits without the so called high.
since they were unable to develop a synthetic form of THC that was as good as natural real THC, Ronald Reagan commissioned Dr. Heath/ Tulane University to kill Rhesus monkies with carbon monoxide poisoning
Damage and Dead Monkeys!
In 1974, California Governor Ronald Reagan was asked about decriminalizing marijuana.
After producing the Heath/Tulane University study, the so-called "Great Communicator" proclaimed, "The most reliable scientific sources say permanent brain damage is one of the inevitable results of the use of marijuana." (L.A. Times)
The report from Dr. Heath had concluded that Rhesus monkeys, smoking the equivalent of only 30 joints a day, began to atrophy and die after 90 days.
And ever since, dead brain cells found in monkeys who were forced to smoke marijuana has been given maximum scare play in federal booklets and government sponsored propaganda literature against pot.
Senator Eastland of Mississippi used it throughout the mid-1970s to horrify and stop national legislators from supporting NORML's decriminalization bills in Congress, mostly sponsored by the late Senator Jacob Javitts of New York.
Reports of the study have also been distributed by the hierarchy of drug rehabilitation professionals as part of their rationalization for wanting to get kids off pot, based on supposed scientific studies.
It is used to terrorize parent groups, church organizations, etc., who redistribute it still further.
Heath killed the half-dead monkeys, opened their brains, counted the dead brain cells, and then took control monkeys, who hadn't smoked marijuana, killed them too, and counted their brain cells.
The pot smoking monkeys had enormous amounts of dead brain cells as compared to the "straight" monkeys.
The public outlook"
Ronald Reagan's pronouncement by trusting the federal research to be real and correct was based on the fact that marijuana smoking was the only difference in the two sets of monkeys.
The reality is Reagan had other motives. Ronald Reagan was part of the brotherhood, “a 33d% Lucifarian Freemason.”
Hemp was a threat to their illegal Federal Reserve, drug trades, cruid oil, petrochemical, pharmiceutical, cancer, synthetic, current agriculture, lumber, cotton, tobacco and automobile endustries and the defamation of corperate America as a whole.
The only way to keep their corupped running intrest was to clasify Hemp as the moat deadly substance under schedule 1.
The government ballyhooed false science to press and PTA, who trusted the government completely.
In 1980, Playboy and NORML finally received for the first time after six years of requests and suing the government an accurate accounting of the research procedures used in the infamous report:
When NORML/Playboy hired researchers to examine the reported results against the actual methodology, they laughed.
The Facts:
Suffocation of Research Animals
As reported in Playboy, the Heath "Voodoo" Research methodology involved strapping Rhesus monkeys into a chair and pumping them with equivalent of 63 Colombian strength joints in "five minutes, through gas masks," losing no smoke.
Playboy discovered that Heath had administered 63 joints in five minutes over just three months instead of administering 30 joints per day over a one- year period as he had first reported.
Heath did this, it turned out, in order to avoid having to pay an assistant's wages every day for a full year.
The monkeys were suffocating! Three to five minutes of oxygen deprivation causes brain damage"dead brain cells."
(Red Cross Lifesaving and Water Safety Manual) With the concentration of smoke used, the monkeys were a bit like a person running the engine of a car in a locked garage for 5, 10, 15 minutes at a time every day!
The Heath Monkey study was actually a study in animal asphyxiation and carbon monoxide poisoning.
Among other things, Heath had completely (intentionally? incompetently?) omitted discussion of the carbon monoxide the monkeys inhaled.
Carbon monoxide, a deadly gas that kills brain cells, is given off by any burning object.
At that smoke concentration, the monkeys were, in effect, like a person locked in a garage with the car engine left running for five, 10, 15 minutes at a time every day!
All subsequent researchers agree the findings in Heath's experiment regarding marijuana were of no value, because carbon monoxide poisoning and other factors were totally left out and had not been considered in the report. This study and others, like Dr. Gabriel Nahas' 1970s studies, tried to somehow connect the THC metabolites routinely found in the fatty tissue of human brains, reproductive organs, and

other fatty areas of the body to the dead brain cells in the suffocated monkeys.
The government passed off the brain cell death rate contributed from carbon monoxide, ah THC in order to squash it.
In 1983 the Reagan Bush administration told universities and researchers to destroy all 1966-76 cannabis research work.
Now, in 1999, 17 years have passed and not a single word of Dr. Heath's or Dr. Nahas' research has been verified!
But their studies are still hauled out by the Partnership for a Drug Free America, the Drug Enforcement Administration, city and state narcotics bureaus, plus politicians and, in virtually all public instances, held up as scientific proof of the dangers of marijuana.
This is U.S. government propaganda and disinformation at its worst!
The public paid for these studies and has the right to the correct information and history being taught in our taxpayer sponsored schools.
In 1996, Gabriel Nahas, in France, sued Mishka, the translator of the French edition of this book, "L'Emperor est Nu!", for damages.
Mishka wrote that Nahas' studies were viewed by the world as garbage.
The French court, upon hearing all the testimony by Nahas, and after Nahas had spent an quivalent of tens of thousands of American dollars on legal fees, awarded him its highest insult: one franc, the equivalent of approximately 15 cents American for damages, and no legal fees!
We only found out about the Nixon, Marijuana, and the Shafer Commission study a few years ago thinks to dedicated medical & law professionals who filed freedom of rights of information request.
The government lied for many reasons, one of the main reason is pharmaceutical companies spend billions every year lobbying to keep cannabis illegal for the fact is they make trillions off cancer drugs & research.
they are already aware that cannabis cures cancer.
They have a grate con going at the moment.
Cancer patients & their loved ones will have to spend their entire life savings or even sell their houses & businesses in order to pay for chemotherapy & other cancer treatment drugs.
A lot of the time they spend & all that money spent, still their loved ones die anyway.
If the public found out that the government had been lying for over 40 years, that millions of lives could have been saved & the dying could grow the cure they needed in their own back yard the public would go ANARCHY.
Smoking cannabis dose not cure cancer, eating, juicing, or eating cannabis oil does.

What is the significance of U.S. government patent number 6 million 630,507.
The U.S. government has a patent on cannabis.
This official government document unequivocally states that cannabinoids are useful in the prevention and treatment of a wide variety of diseases including autoimmune disorders, Stroke, Trauma, Parkinson's, Alzheimer's, HIV, Dementia, cancer and Nausea,
The patent, awarded in 2003 is based on research done by the national institute of health and is assigned to the U.S. department of health and human services.
So! Why is this important?
here is a legal document in the public domain, which flies in the face of the U.S. government's stated position with regard to the classification of cannabis as a scheduled 1 substance having no currently excepted medical use.
Spread word of this to everyone you know.
Citing this pattern will stop the medical marijuana is a myth advocates dead in their tracts.
They simply cannot argue with it. The forces that will keep cannabis illegal are vocal and well funded, but they are not impervious to persistent effort. The lynchpin in the war on drugs is cannabis.
Without the suppression and interdiction of this popular and widely used substance, have simply would not be enough for legal drug used going on to justify the huge amount of money and resources spent on fighting drugs.
I believe disseminating this information to as many people as possible is a crucial strategy in loosening the lynchpin's stranglehold in order to awaken public conscious perception on the fundamental importance of safe aguaculture, ecological, industrial hemp and medical cannabis.
The war on drugs is a failed and destructive policy, marijuana smokers are not a threat to society.
One day, the existence of this irrefutable patent will become widely held public knowledge and the government's rhetoric will be shown to be as hollow as a busted drum.
A world where alcohol is a perfectly legal substance attainable almost everywhere yet cannabis users are outlawed in present of their possessions stolen by the courts; quite simply: offense reason!

And Today Big
Pharma Are Making Billions....
Millions Are Dying From Their Drugs.... And The Rockefeller's.... Well They Are Part Of The New World Order (LUCIFERIANS ILLUMONATI)
Millions Are Dying In Plain Sight As Well Helps Their Population Reduction Plan.
Although David Rockefeller just passed away his families legacy of depopulation plan will carry on unless we do something about it.
you possess the power to impact the global narrative & to be part of the history

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The Endocannabinoid System

Obesity, particularly abdominal obesity and the associated cardio metabolic complications are critical areas of investigation. The endocannabinoid system helps to regulate the central control of energy balance and peripheral metabolic processes, both of which may contribute to cardio metabolic risk factors ENDOCANNABINOID SYSTEM an overview. The endocannabinoid system or ECS influences multiple physiologic processes. This intricate system moderates energy intake as well as nutrients transport, metabolism and storage. The ECS regulates these processes through andodgelis ligands such as anandamide, 2 erekidonal glycerol and the CB1 receptor. CB1 receptors are located in the brain, digestive tract, muscle and adipose tissue. integration of these central and peripheral ECS components is achieved through neuronal and hormonal singling. ENDOCANNABINOID BIOLOGY Within the brain CB1 receptors are among the most abundant G protein coupled receptors, however in contrast to classical singling where information travails from pre to post

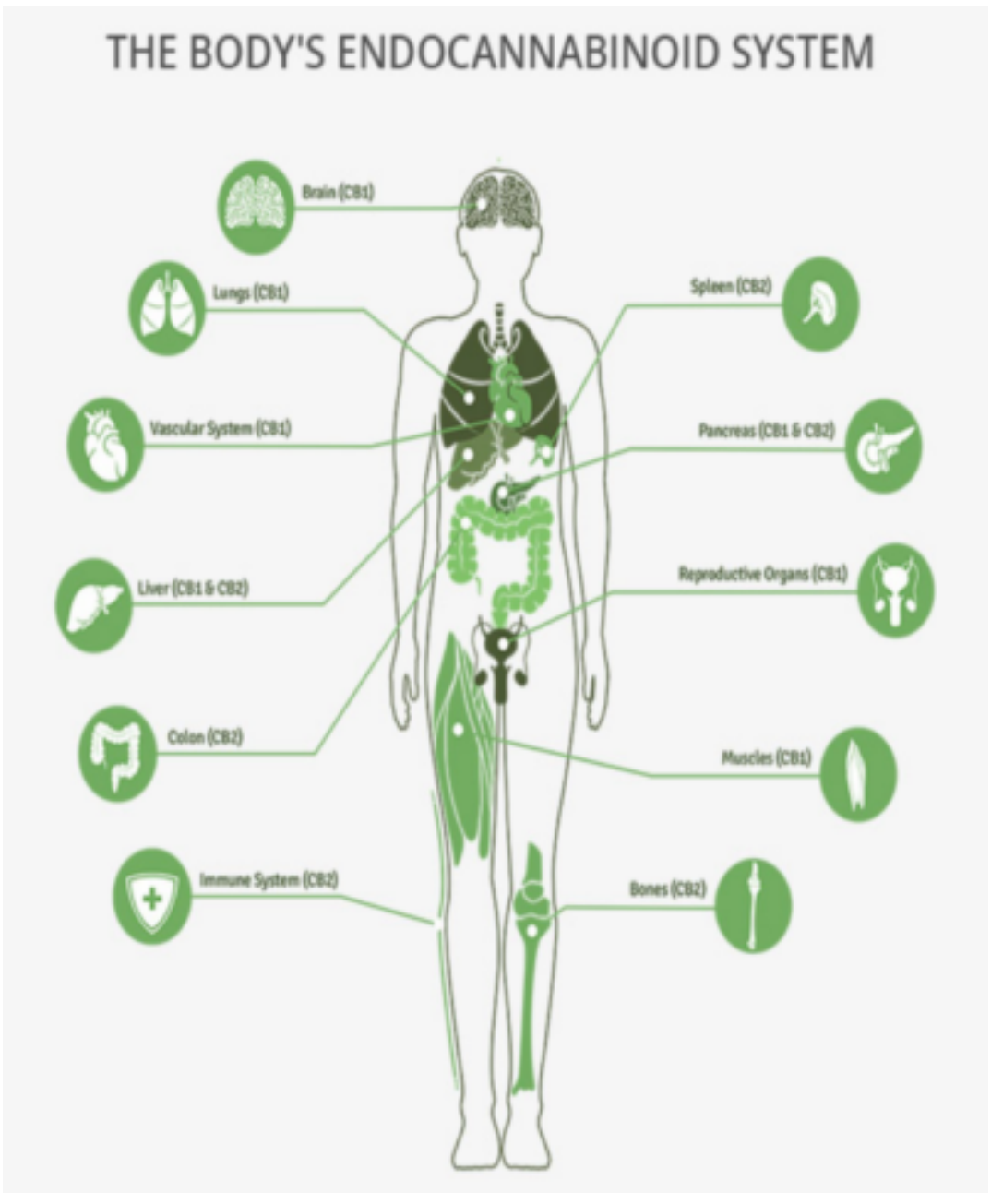
Synaptic neurons the ECS uses retrograde singling. The information travails from post to pre synaptic neuron. Let's take a closer look at this mechanism using a glutamate turgic neuron model. When in action protintural reaches the axiom terminal maim brain depolarization triggers the release of glutamate. Glutamate binds to post synaptic glutamate receptors inducing calcium channels to open. During periods of intense neural activity, calcium accumulates in the post synaptic neuron. This calcium buildup causes the synthesis and release of endocannabinoids from maim brain lipids. Diffusing across the synaptic cleft, the cannabinoids bind to the CB1 receptor activating the G protein. Activation influences ion flow. The result, suppression of pre synaptic neural transmitter release endocannabinoids are subsequently taken back into the cell and intermatically degraded. In addition to acting as neural messengers ENDOCANNABINOIDS mediate paracrine and autocuing signaling in adipocyte's podocyte's and other cells. ENDOCANNABINOID SYSTEM APPETITE, MOTIVATION AND SATIETY endocannabinoid system activity in the central nerves system regulates food intake. for example ECS stimulation by hunger and fasting singles, stimulates apatite and increases the palatability of food. endocannabinoids slow gastric emptying and GI transit and appear to stimulate secretion of ghrelin a neural peptide that increases apatite and food intake. After eating polosistic quinine in the droning triggers satiety signals. Subsequently ECS activity is decreased through suppression of CB1 expression. An increase in the adiposity hormone lepton decrease endocannabinoid levels in the athepatimos and decreases food intake. ENDOCANNABINOID SYSTEM PERIPHERAL METABOLIC REGULATION ECS regulation of peripheral metabolism influences energy balance. Stimulation of the ECS increases food intake and adiposity. Conversely blocking CB1 receptors reduces food intake and adiposity. In the liver ECS stimulation can lead to lipogenesis through the activation of hepatic lipogenic enzymes and increase fatty acid synthesis. ENDOCANNABINOID SYSTEM DYSLIPIDEMIA AND GLUCOSE HOMEOSTASIS Chronic stimulation of the ECS is associated with dyslipidemia. Activation of CB1 receptors increases expression of SREDP1C a lipogenic transcription factor and increase fatty acid synthesis. SREDP1C increases production of lipogenic enzymes ACC1 and fatty acid synthase. Increase fatty acid synthesis can lead to production of large triglyceride rich VLDL. Large triglyceride rich VLDL sets the stage for the atherogenic lipid profile of small dense LDL, decreased levels of apron protective HDL and overall increases in cholesterol and triglycerides levels. Adiponectin, another hormone secreted by adipocytes regulates lipid and glucose metabolism. Adiponectin is believed to regulate fatty acid oxidation in muscle and liver thus improving insulin sensitivity. CB1 receptor stimulation in adipocytes reduces Adiponectin, while CB1 blockade increases Adiponectin synthesis. Metabolic deregulation leads to a constellation of symptoms including abdominal obesity, atherogenic dyslipidemia, hypotension, insulin resistance, prothrombotic state and proinflammatory state. As basic and clinical research progresses we will continue to increase our understanding of the central and peripheral endocannabinoid system and it's roll in the regulation of metabolic function.

In the normal transmission of pain ascending nose septic singles travail up the spinal caloric pathway to the thalamus where they are processed and relayed to cortical and other areas. The ascending pathway also relays to the Periaqueductal gray matter, an important part

of the descending pain modulating system where specific cannabinoid receptors are located. Stimulation of the Periaqueductal gray is known to produce an analgesic effect, however this neuroregulatory proses is thought to be tonically restricted by inhibitory gabaergic off cells. Ascending pain signals relay through the Periaqueductal gray suppress the activity of gabaergic off cells through cannabinoid modulation thereby increasing activity in the descending pain modulation pathway and increasing the analgesic effect. This is called depolarization induced suppression of inhibition. A synaptic feedback mechanism is believed to be an inherent part of our normal neuroregulatory processes Including descending pain modulation. In many regions of the CNS including the Periaqueductal gray the generation of postsynaptic action potentials is modified by an endogenous cannabinoid regulatory system. Endogenous cannabinoids are synthesize on demand from the phospholipid bilayer of the post synaptic midbrain. These lipophilic ligands are released directly into the synaptic cleft and act in retrograde fashion on the presynaptic neuron where the cannabinoid receptors are expressed. Binding of the endogenous cannabinoids effects intracellular signal transduction pathways reducing the influx of calcium ions into the presynaptic neuron causing a decrees in further neurotransmission release. This intern influences the frequency of post synaptic firing. The administration of exogenous cannabinoids orally work through inhalation may therefore provide an option for treating pain that has responded parley to conventional pain therapy. When exogenous cannabinoids are administered orally they travail veal the GI tract to the liver where they are metabolized before entering the blood stream and then the CNS. The onset of action from oral administration is less rapid compared to the inhaled route. When exogenous cannabinoids are inhaled they travail through the respiratory tract to the alveoli of the lung where they defuse rapidly into the circulatory system. They thin travail through the blood stream toured the CNS where they defuse across the blood brain barrier and into the surrounding tissues of the brain and spinal cord. Exogenous cannabinoids exert their modulatory effects upon the abundant cannabinoid receptors distributed throughout the CNS. The effects of cannabinoids may be illustrated by comparing three normal synapses showing normal pain transmission, the influences of endogenous cannabinoids and the influence of exogenous cannabinoid administration. Exogenous cannabinoids from the blood stream bind to cannabinoid receptors on the presynaptic neuron and mimic the endogenous synaptic modulatory effect by decreasing influx of calcium ions in this way pain and other neural processes are modulated. The clinical effects of exogenous cannabinoid administration are not limited to pain and have proven effects including the reduction of anxiety, spasticity, anorexia and nausea. The development of normal drugs to modulate endogenous cannabinoid reuptake in metabolism may also prove beneficial in pain therapy. The therapeutic benefits of cannabinoids including the modification of the endogenous cannabinoid singling system should be further explored through clinical research

The EC system consists of cannabinoid receptors, the endocannabinoids, and all the factors involved in the synthesis and degradation of endocannabinoids.
Pineal, Hypothalamus, Pituitary Thyroid, Parathyroids, Thymus, Adrenals, Pancreas, Ovary, Testes.
They claim it was established at least 600 million years ago. The EC system has a slightly different function in vertebrates and in invertebrates. A survey of amphibians and birds has shown that the ECB signaling acts as a kind of behavioral switch that allows the redirection of the less meaningful to the more urgent types of behavior in case of emergency adjustment to geoclimatic changes. The EC system has contributed significantly to the development of most living creatures as it began to develop simultaneously with the first living creatures, it's development is very diversified and interwoven with various animals and plant species.
Peripheral nervous system, cranial nerves, spinal nerves.
Cannabinoids are an essential element of the suction reflex in infants and are associated with digestive reflexes and appetite. Pharynx, Oral Cavity, Tounge, Salivary, Glands, Parotid, Sublingual, Submandibular, Esophagus, Diaphragm, Liver Gallbladder, Stomach, Pancreas, Pancreatic Duct,
Common Blue Duct, Duodenum, Transverse Colon, Ascending Colon, Cecum, Appendix, Small Intestines, Descending Colon, Rectum, Anal Canal, Anus.
THC and endocannabinoids reduce the parasympathetic function and the vomiting reflex. Cannabinoids. Also protect the heart by reducing high blood pressure.
Aorta, Superior Vena Caval, Pulmonary Arteries, Inferior Vena Cava, Descending Aorta, Femoral Vein, Femoral Artery.
The EC system is a vital element in shaping the corticothalamic synaptic circuit in embryos.
Orbital Gyri, DM Cingulate, Gyrus, ATM Striatum, Frontal Gyri, Precentral Gyrus, Postcentral Gyrus, VN Temporal Gyri, Parietal Gyri, CGN, TRN, NGN.
The use of hemp promotes neurogenesis, the formation and growth of new brain cells in hippocampus, which is related to antianxiolitic, antidepressant effects and the feeling of euphoria. Hemp has the most concentrated external source of cannabinoids, that are essential for the proper functioning of our body, Action Potential 2-AG e CB production Ca2 AMPA receptors and play a key role in embryonic development of the brain, the formation of brain cells and synapses.
Dopamine terminal, dopamine, dopamine receptors, THC, THC receptor terminal, THC receptors,
Concentrations of CB1 receptors
Cerebellum1 Movement, Basal Ganglia1 Movement, Cerebral Cortex1 Higher cognitive function, Hippocampus1 Learning, memory, stress, Spinal Cord1 Peripheral sensation including pain, Hypothalamus2 Appetite, Medulla3,4 Nausealvomiting, chemotherapy trigger zone (CTZ).
Formation of new brain cells in adults is crucial for all healing processes.
Cerebral Cortex, Pineal Gland, Suprachiasmatic Nucleus, Optic Chiasm, Pituitary, hypothalamus.
The cannabinoid receptors are condensed in the brain and spinal cord, but mostly in the hippocampus, amygdala, Cerebral Cortex, and pineal gland, Medial Orbito-Fruntal, Frontal Pole, Superior Frontal, Paracentral lobule, Precuneus, Cuneus, Lingual, Fusiform, Rostral Anterior Cingulate, Caudal Anterior Cingulate, Isthmus, Temporal Pole , Entorhinal, Parahippocampal, Pericalcarine
especially in the frame of cortico-talamic synaptic circuits responsible for awareness.

Parietal (Attentional Expression), Frontal (Attentional control) Thalamus.
The EC system is instrumental in shaping synaptic connections between the cerebral cortex and ganglia, which are responsible for targeted and reasonable behavior,
Basal Ganglia, Thalamus, Amygdala, Nucleus Accumbens,
VYA, Frontal Cortex, Ventral pallidum, Stratum, Substantia Nigra,
Cerebellum, Hippocampus, Brain Stem,
while disruption of these connections are the cause of many mental disorders. (Pineal Gland)
Cannabinoids are responsible for the transmission of visual stimuli from the pineal gland to the cortex, and also have an important role in recognizing and identifying visual impulses and pictures.
Cerebellum, Midbrain, Corpus Callosum, Thalamus,
Hypothalamus, Pituitary Gland Mammillary Body, Pons, Medulla oblongata, Spinal Cord,
hieroglyph, eye of Horus (Corpus Callosum, Thalamus, Medulla Oblongata Hypothalamus)
The EC system is controlled by an electrical frequency of the pineal gland (thalamus), which is essential to maintain the balanced functioning of an organism,
while disturbances in the electrical functioning of the pineal gland cause seizures. The EC system is involved in the functioning of the entire body. Organs Of the Immune System.
Tonsils and adenoids, Lymph nodes, lymphatic vessels Thymus armpit Lymph Nodes Spleen,
Appendix, Peyer's Patches Lymph nodes in legs, Lymphatic Vessels, bone marrow.
One of the tasks of endocannabinoids is the defense system against excitotoxicity,
Viruses, Toxins, carcinogens Funguses Pollution, Bacteria, Germs, Parasites.
Disturbances in the EC system are reflected as different nerve diseases.
Jaka has noticed that there are similarities of the endocannabinoid and the biophotonic system. The biophotonic system is namely our electromagnetic field, consisting of photons, weak wave-particles of the visual spectrum. According to the energy theory, the photons are the building blocks of the universe, originating in time before there were any elements or atoms. Photons have a remarkable ability to connect and form frequency nets. Their communication is instant regardless of distances. Photons can also save information as memories. Large quantities of photons can be stored in crystals for unlimited time and activated later or transformed into another kind of energy. Both ECS and PBS, are concentrated mainly in the brain and spine cerebellum, Corpus callosum body of fornix, pituitary gland, especially in the thalamus and the cortex. Photonic therapy has much milder, though similar effect as cannabis therapy, namely antidepression and neurogenesis. While the ECS is responsible for the short term memory, the BPS is responsible for the long term memory. Jaka thinks it is no coincidence, that THC, CBD, CBN and all the other 85 known cannabinoids are concentrated in the hemp crystals, as photonic energy can concentrate in crystals and the ECS can in essence be viewed as energy transfer between the subatomic level and the molecular level.



THE ENDOCANNABINOID SYSTEM: PHYSIOLOGY AND PHARMACOLOGY

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FERNANDO RODRÍGUEZ de FONSECA, **IGNACIO DEL ARCO**, **FRANCISCO JAVIER BERMUDEZ-SILVA**, **AINHOA BILBAO**, **ANDREA CIPPITELLI**, **MIGUEL NAVARRO**

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Abstract

The endogenous cannabinoid system is a ubiquitous lipid signalling system that appeared early in evolution and which has important regulatory functions throughout the body in all vertebrates. The main endocannabinoids (endogenous cannabis-like substances) are small molecules derived from arachidonic acid, anandamide (arachidonoylethanolamide) and 2-arachidonoylglycerol. They bind to a family of G-protein-coupled receptors, of which the cannabinoid CB1 receptor is densely distributed in areas of the brain related to motor control, cognition, emotional responses, motivated behaviour and homeostasis. Outside the brain, the endocannabinoid system is one of the crucial modulators of the autonomic nervous system, the immune system and microcirculation. Endocannabinoids are released upon demand from lipid precursors in a receptor-dependent manner and serve as retrograde signalling messengers in GABAergic and glutamatergic synapses, as well as modulators of postsynaptic transmission, interacting with other neurotransmitters, including dopamine. Endocannabinoids are transported into cells by a specific uptake system and degraded by two well-characterized enzymes, the fatty acid amide hydrolase and the monoacylglycerol lipase. Recent pharmacological advances have led to the synthesis of cannabinoid receptor agonists and antagonists, anandamide uptake blockers and potent, selective inhibitors of endocannabinoid degradation. These new tools have enabled the study of the physiological roles played by the endocannabinoids and have opened up new strategies in the treatment of pain, obesity, neurological diseases including multiple sclerosis, emotional disturbances such as anxiety and other psychiatric disorders including drug addiction. Recent advances have specifically linked the endogenous cannabinoid system to alcoholism, and cannabinoid receptor antagonism now emerges as a promising therapeutic alternative for alcohol dependence and relapse.

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INTRODUCTION

Twenty-four years of pharmacological research separate the identification of the main psychoactive constituent of Cannabis sativa preparations, (−)-Δ9-tetrahydrocannabinol (THC) (Gaoni and Mechoulam, 1964; Mechoulam, 1970) from the characterization (Devane et al., 1988; Herkenham et al., 1991) and molecular cloning (Matsuda et al., 1990) of its cellular target, the cannabinoid CB1 receptor (CB1). The extensive research on the structure and activity of the natural constituents of Cannabis (termed cannabinoids) and the development of synthetic compounds with high potency and stereoselectivity have led to the identification of the main physiological functions that are modulated by this new class of drugs (Howlett et al., 1990). The discovery of the cannabinoid receptor and the availability of highly selective and potent cannabimimetics led to the rapid identification of a family of lipid transmitters that serve as natural ligands for the CB1 receptor: arachidonoylethanolamide (AEA), named anandamide from the Sanskrit ‘internal bliss’ (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). The pharmacological properties of the endocannabinoids were found to be very similar to those of the synthetic cannabimimetics. The subsequent description of a complex biochemical pathway for the synthesis, release (Di Marzo et al., 1994; Cadas et al., 1996), transport (Beltramo et al., 1997) and degradation (Cravatt et al., 1996) of endocannabinoids completed the scaffold of a new signalling system termed the ‘endocannabinoid system’. Since the discovery of anandamide, more than 3500 scientific reports have comprehensively explored the main aspects of the endocannabinoid system. This system now appears as a relevant modulator of physiological functions not only in the central nervous system but also in the autonomic nervous system, the endocrine network, the immune system, the gastrointestinal tract, the reproductive system and in microcirculation (Di Marzo et al., 1998; Table 1).

Table 1.A selection of reviews and reports that explore in depth the main aspects of endocannabinoids and their receptors

Topic	References
Biochemistry and molecular biology	Matsuda, 1997; Felder and Glass, 1998; Giuffrida et al., 2001; Piomelli, 2003
Signalling	Schlicker and Kathmann, 2001; Wilson and

	Nicoll, 2002; Freund et al., 2003
Anatomy and development	Breivogel and Childers, 1998; Fernandez-Ruiz et al., 2000; Elphick and Egertova, 2001
Physiology	Di Marzo et al., 1998; De Petrocellis et al., 2004
Pharmacology	Howlett et al., 2002
Addiction	Maldonado, 2002; Maldonado and Rodríguez de Fonseca, 2002; Tanda and Goldberg, 2003; Martin et al., 2004
Pain	Calignano et al., 2000; Pertwee, 2001
Behaviour	Rodríguez de Fonseca et al., 1998; Chaperon and Thiebot, 1999; Castellano et al., 2003
Gastrointestinal system	Izzo et al., 2001
Immune system	Cabral, 2001
Cardiovascular system	Kunos et al., 2002; Randall et al., 2002; Hiley and Ford, 2004
Therapeutic applications	Piomelli et al., 2000; Cravatt and Lichtman, 2003; Guzman, 2003; Smith et al., 2004

Since the discovery of anandamide in 1992, over 3500 publications have reported new data on the biological role of the endogenous cannabinoid system.

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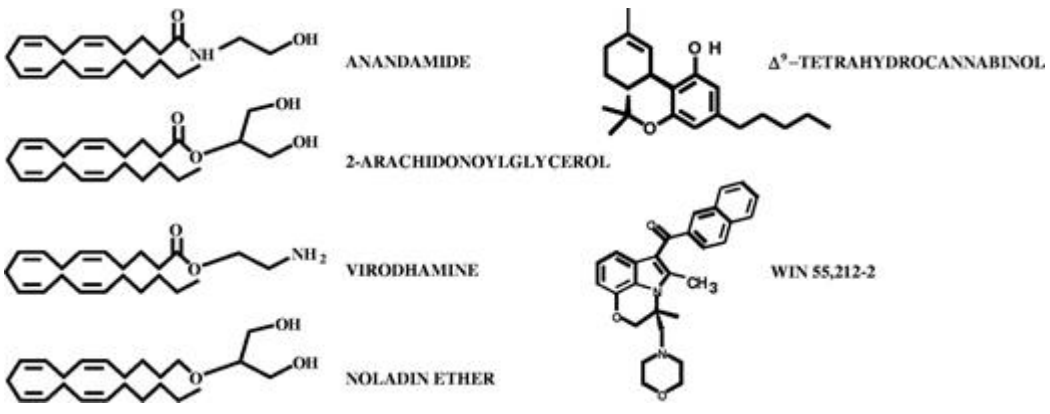
The present review gives a general perspective of the endogenous cannabinoid system, including the main pharmacological advances in the development of drugs capable of modulating their dynamics. The review focuses on the role of endocannabinoids as modulators of reward circuits and motivated behaviour that are relevant for drug addiction, including alcoholism. In light of the extensive research over the past 12 years, several specialized reviews wherein the reader will find a more profound analysis of the role played by the endocannabinoid system in selected physiological functions are shown in Table 1.

Biochemistry of the endogenous cannabinoid system

Endocannabinoids. When discovered, the endocannabinoids were found to be derivatives of arachidonic acid, which resembled other lipid transmitters (eicosanoids such as prostaglandins or leukotrienes). Additional studies revealed the existence of other structure-related lipid messengers including palmitylethanolamide or oleoylethanolamide, which are not active at cannabinoid receptors. These messengers will not be included in this review, although they serve important physiological functions in inflammation, pain control, feeding behaviour and lipid metabolism (Calignano et al. 1998; Rodríguez de Fonseca et al., 2001; Fu et al., 2003; Piomelli, 2003).

Endocannabinoids are derivatives of arachidonic acid conjugated with ethanolamine or glycerol. Figure 1 depicts the chemical structure of four endocannabinoids, anandamide, 2-arachidonoylglycerol (2-AG), the ester of arachidonic acid and ethanolamine; virodhamine which resembles anandamide (Porter et al., 2002), and the 2-arachidonyl glyceryl ether noladin, an analogue of 2-AG (Hanus et al., 2001). All these endocannabinoids have been found in the brain, plasma and peripheral tissues, although the relevance of noladin has been questioned recently (Oka et al., 2003) because its concentration in the brain is too low for this compound to act as an endogenous cannabinoid receptor ligand. In the brain, the concentration of anandamide is 200-fold lower than that of 2-AG (Sugiura et al., 1995; Stella et al., 1997). The monoglyceride 2-AG is a metabolic intermediate in lipid metabolism whereas anandamide is the product of the cleavage of a membrane phospholipid. However, after depolarization or receptor stimulation (e.g. dopamine D2 receptor-mediated), the concentration of anandamide can rise up to 5–12 fold in a time-limited fashion (Giuffrida et al., 1999; Stella and Piomelli, 2001; Kim et al., 2002).

Fig. 1.



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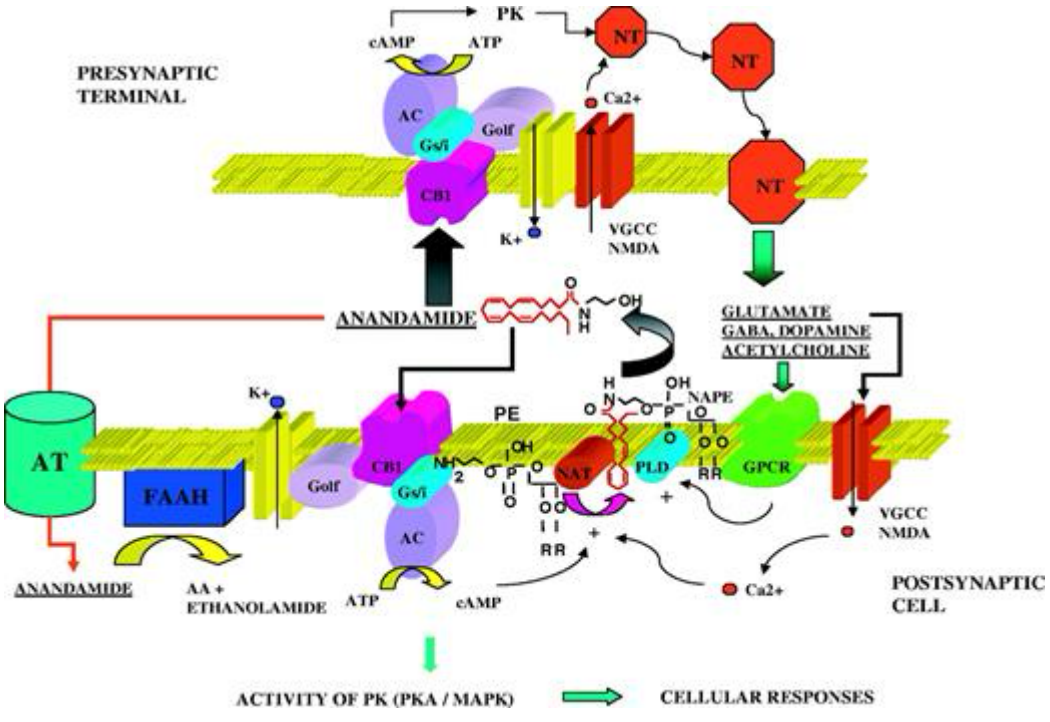
Cannabinoid receptor agonists. Left, the structure of four arachidonic acid derivatives that have been identified as endogenous ligands for both the cannabinoid CB1 and CB2 receptors. Right, the structure of Δ9-tetrahydrocannabinol (THC), the main cannabinoid receptor agonist present in Cannabis preparations and that of the aminoalkylindole WIN-55,2122, a synthetic cannabinoid receptor agonist active at CB1 and CB2 receptors.

Synthesis and release. Different pathways are involved in the synthesis and release of anandamide and 2-AG. Figure 2 shows the dynamics of formation and degradation of anandamide.

Fig. 2.



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Overview of the biochemical pathways for synthesis, degradation and cellular actions of the endogenous cannabinoid anandamide. Anandamide is released from a membrane lipid precursor (N-arachidonoyl-phosphatidylethanolamine, NAPE) by the action of a specific phospholipase D (PLD) activated by depolarization or G-protein-coupled receptor (GPCR) stimulation. NAPE biosynthesis is catalysed by a membrane enzyme, N-acyltransferase (NAT) activated by calcium (Ca2+) and cAMP. Anandamide acts as a retrograde messenger at presynaptic cannabinoid receptors (CB1), where it regulates neurotransmitter release (NT) through its second transduction systems [mainly Ca2+ incorporated through voltage-gated calcium channels (VGCC) or glutamate NMDA (N-methyl-D-aspartate) receptors]. Anandamide also acts as a neuromodulator of major transmitter systems, including dopamine, at postsynaptic cells, where it regulates excitability and synaptic plasticity through its modulation of potassium (K+) channels, and the regulation of a broad spectrum of protein kinases (PK) including protein kinase A and mitogen-activated protein kinases (MAPK). Anandamide action is terminated through a two-step process, which includes, first, its cellular uptake through a specific anandamide transporter (AT) and second, degradation by enzymatic cleavage to arachidonic acid (AA) and ethanolamide by the membrane-bound enzyme fatty acid amidohydrolase (FAAH).

Anandamide is formed by the cleavage of a phospholipid precursor, the N-arachidonoyl-phosphatidylethanolamine (NAPE). The precursor is synthesized by the enzyme N-acyltransferase (NAT), which catalyses the transfer of arachidonic acid from phosphatidylcholine to the head group of phosphatidylethanolamine. This enzyme requires the presence of Ca2+ and is regulated by cAMP, which enhances the activity of NAT by phosphorylation mediated through the cAMP-dependent activity of protein kinase A (Cadas et al., 1996; Piomelli, 2003). The release of anandamide from NAPE is catalysed by a specific phospholipase D (PLD), which has been cloned recently (Okamoto et al., 2004). This enzyme has no homology with the known PLD enzymes and is classified as a member of the zinc metallohydrolase family. Its presence is highest in the brain, kidneys and testis. The activity of PLD is regulated by depolarization or by activation of the ionotropic glutamate N-methyl-D-Aspartate (NMDA) receptors or nicotinic α7 neuronal receptors (Stella and Piomelli, 2001; Piomelli, 2003) or stimulation of the metabotropic receptors of major neurotransmitters including dopamine, glutamate and acetylcholine (Giuffrida et al., 1999; Varma et al., 2001; Kim et al., 2002).

The synthesis and release of 2-AG is different from that of anandamide. Because 2-AG is a monoglyceride, its formation is closely associated with the metabolism of triacylglycerol, mainly by the receptor-dependent activation of phosphatidylinositol-specific phospholipase C (PLC). The standard model proposes that activation of metabotropic receptors coupled to the PLC and diacylglycerol (DG) lipase pathway will systematically lead to increases in 2-AG production (Stella et al., 1997; Piomelli, 2003). Cloning of the enzyme 1,2-diacylglycerol lipase (Bisogno et al., 2003) has confirmed this hypothesis, as well as the contribution of ionotropic purinergic receptors such as P2XT, which boosts 2-AG formation (Witting et al., 2004). Although 2-AG formation is dependent on Ca2+, its regulation is independent

of anandamide synthesis and release. Once anandamide and 2-AG are formed, they target the CB1 receptors in the same cell where they were formed, via diffusion within the plasmalemma, or they can be released to the extracellular fluid where they reach distant targets (i.e. presynaptic terminals) with the apparent help of protein carriers such as lipocalins or albumin (Piomelli, 2003).

Uptake and degradation. Endocannabinoid signalling is terminated by a two-step process that includes transport into cells and hydrolysis by two specific enzymatic systems. Both steps exert a tight control of endocannabinoid levels in tissues, rapidly eliminating these signalling molecules. Endocannabinoid uptake is mediated by a transporter (Beltramo et al., 1997), which is widely distributed throughout the brain (Giuffrida et al., 2001). The transporter is an elusive molecule which works in a manner that is similar to other lipid carriers: it facilitates the uptake of both anandamide and 2-AG in an energy-independent fashion (Beltramo et al., 1997). The anandamide transporter is saturable, displays substrate specificity and can be blocked by specific drugs such as AM 404 (Fig. 4). A major issue of debate has been the potential coupling of endocannabinoid transport and degradation: it is possible that the energy for the uptake process is obtained by its coupling to the enzymatic hydrolysis of anandamide. However, a recent report seems to confirm that transport and degradation are independent processes (Fegley et al., 2004). The degradation of endocannabinoids is performed by two specific enzymatic systems: the fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996) and the monoacylglyceride lipase (MAGL) (Dinh et al., 2002). FAAH is a membrane enzyme that belongs to the serine–hydrolase family. FAAH is widely distributed throughout the body, with high concentrations in the brain and liver. FAAH can degrade many fatty acid amides, including acylethanolamides such as anandamide and the sleep factor oleamide. Although FAAH can inactivate 2-AG, the main enzyme responsible for the inactivation of this monoglyceride is MAGL (Dinh et al., 2002). This enzyme is also a serine hydrolase and its distribution in the nerve terminals of specific brain neurons has been determined recently (Gulyas et al., 2004).

Receptors. Two major cannabinoid receptors have been cloned, both of which belong to the superfamily of G-protein-coupled receptors. The first receptor described was named the CB1 receptor and it is mainly located in the terminals of nerve cells (central and peripheral neurons and glial cells), the reproductive system (i.e. testis), some glandular systems and the microcirculation (Devane et al., 1988; Howlett et al., 1990; Herkenham et al., 1991; Wagner et al., 1997; Batkai et al., 2001). The CB2 cannabinoid receptor was found initially in multiple lymphoid organs with the highest expression detected in B lymphocytes, moderate expression in monocytes and polymorphonuclear neutrophils and the lowest expression in T lymphocytes, although subsequent studies identified it in microglial cells as well (Munro et al., 1993; Galiègue et al., 1995; Piomelli, 2003). An interesting aspect of cannabinoid receptors is their expression during development of the brain, where they control cell differentiation (Rueda et al., 2002), and their presence in tumour cells derived from glial cells and the main epithelia (Galve-Roperh et al., 2000; Sanchez et al., 2001; Casanova et al., 2003). Pharmacological studies revealed the existence of other endocannabinoid targets including the vanilloid receptor (Zygmunt et al., 1999) and at least two non-CB1 non-CB2 'CB-like' receptors, one in the vascular bed and the other in glutamatergic axon terminals (Hajos et al., 2001; Howlett et al., 2002; Kunos et al., 2002). The existence of these and other putative cannabinoid receptors, and their role in endocannabinoid physiology can be clarified only after their molecular characterization. Cannabinoid receptors, especially the CB1 receptor, display unique properties. The most relevant property is their preservation throughout evolution: e.g. human, rat and mouse CB1 receptors have 97–99% amino acid sequence identity. The preservation of this ancient signalling system in vertebrates and several invertebrate phyla reflects the important functions played by the endocannabinoids in cell and system physiology. A second remarkable characteristic of the CB1 receptors is their high expression in the brain. The CB1 receptor is the most abundant G-protein-coupled receptor, with densities 10–50 fold above those of classical transmitters such as dopamine or opioid receptors (Howlett et al., 1990; Herkenham et al., 1991). Another important characteristic is the low efficiency of CB1 receptor coupling to its transduction system: e.g. when compared with opioid receptors, CB1 receptors are 7-fold less efficient in their ability to couple to G proteins (Breivogel et al., 1998; Felder and Glass, 1998; Manzanares et al., 1999).

Both cannabinoid receptors are coupled to similar transduction systems. Cannabinoid receptor activation was initially reported to inhibit cAMP formation through its coupling to Gi proteins (Devane et al., 1988; Howlett et al., 1990), resulting in a decrease of the protein kinase A-dependent phosphorylation processes as well. However, additional studies found that the cannabinoid receptors were also coupled to ion channels through the Golf protein, resulting in the inhibition of Ca2+ influx through N (Mackie and Hille, 1992), P/Q (Twitchell et al., 1997) and L (Gebremedhin et al., 1999) type calcium channels, as well as the activation of inwardly rectifying potassium conductance and A currents (Mackie et al., 1995; Childers and Deadwyler, 1996). These actions are relevant to the role of cannabinoids as modulators of neurotransmitter release (Schlicker and Kathmann, 2001) and short-term synaptic plasticity (Wilson and Nicoll, 2001), as discussed below. Further research also described the coupling of CB1 and CB2 receptors to the mitogen-activated protein kinase cascade, to the phosphatidylinositol 3-kinase, to the focal adhesion kinase, to ceramide signalling and to nitric oxide production (Derkinderen et al., 1996; Bouaboula et al., 1997; Molina-Holgado et al., 1997; Galve-Roperh, 2000; Howlett et al., 2002). Finally, recent studies revealed that under certain conditions, the CB1 receptors can stimulate formation of cAMP by coupling to the Gs protein (Felder et al., 1998).

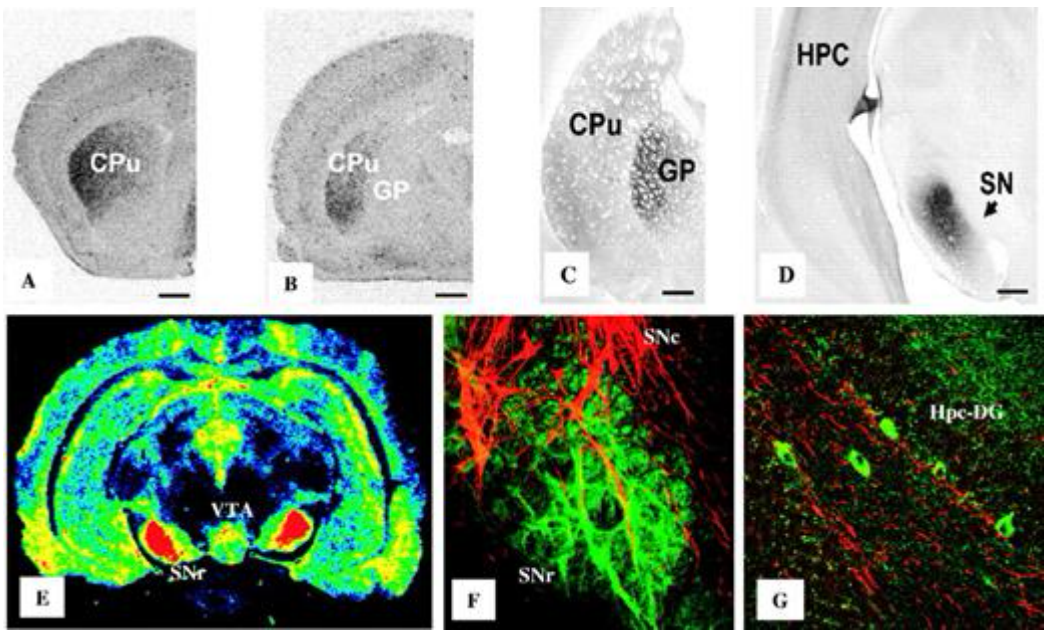
Endocannabinoids exhibit different binding properties and intrinsic activity at CB1 and CB2 receptors. Anandamide behaves as a partial agonist at both CB1 and CB2 receptors, but has higher affinity for the CB1 receptor (Hillard et al., 1999; Howlett et al., 2002). The intrinsic activity of anandamide at CB1 receptors is 4–30 fold higher than at CB2 receptors. However, 2-AG is a complete agonist at both CB1 and CB2 receptors and it exhibits less affinity than anandamide for both CB1 and CB2 receptors (Stella et al., 1997; Howlett et al., 2002).

Functional neuroanatomy of the endogenous cannabinoid system

As described above, the endogenous cannabinoid system is widely distributed throughout the body. In the peripheral tissues the localization of the elements of the endogenous cannabinoid system reflects the distribution of the cell types where they are located (e.g. B lymphocytes in spleen and lymph nodes). However, in the nervous system the distribution is much more complex and structured, and clearly reflects the importance of this system in synaptic transmission. In some regions, such as the hippocampus, there is a complementary distribution of cannabinoid receptors, endocannabinoid transporters and degradation enzymes. However, in other areas of the brain, for instance the thalamus, there are discrepancies (i.e. transport activity and MAGL expression in the absence of a relevant presence of the CB1 receptors) in its distribution, which reflects the gaps in our knowledge of the composition of the endocannabinoid system.

Receptors. From the early work of Herkenham et al. (1991) it was clear that the CB1 receptor distribution was unique among G-protein-coupled receptors, not only because of the very high densities of cannabinoid binding sites but also because of the dynamics of CB1 receptor synthesis and transport. Binding studies and in situ hybridization analysis showed that the cannabinoid receptors are synthesized in somata and the protein transported to axon terminals (Herkenham et al., 1991; Matsuda et al., 1993). The phenotype of the CB1 receptor-expressing neurons corresponds mainly to GABAergic neurons including cholecystokinin-containing neocortical, amygdalar and hippocampal neurons and dynorphin- and substance P-expressing medium spiny neurons of the outflow nuclei of basal ganglia (Tsou et al., 1999; Julian et al., 2003). Several glutamatergic and cholinergic telencephalic and cerebellar neurons also express the CB1 receptors (Piomelli, 2003). In the peripheral nervous system, the CB1 receptors are located in sensory neurons of the dorsal root ganglia. Figure 3 shows how the CB1 receptors are synthesized in medium spiny neurons of the caudate-putamen and the protein transported to the axon terminals in the globus pallidus and substantia nigra. The dense presence of CB1 binding sites in the cerebellum, hippocampus, striatum, globus pallidum and substantia nigra clearly reflects this biological characteristic of CB1 receptors.

Fig. 3.



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Imaging cannabinoid CB1 receptor in circuits of the rat brain reward system. Cannabinoid receptors are mainly located at presynaptic axon terminals. In the basal ganglia, CB1 receptor mRNA expression (panels A and B) is located mainly in GABAergic projecting neurons of the caudate-putamen (CPu), but not in the target nuclei, the globus pallidus or the substantia nigra (GP and SN). However, the protein is mainly detected by immunohistochemistry (panels C and D) in the axon terminals innervating both outflow nuclei of the basal ganglia. Panel E shows the dense presence of CB1 receptors in the substantia nigra and ventral tegmental area (VTA) as mapped by CB1 receptor agonist-stimulated GTP-γ-S incorporation. In these areas, CB1 receptors are not located in dopaminergic neurons (Panel F): confocal imaging using specific antibodies against CB1 receptors (green) and tyrosine hydroxilase (red) shows the compartmentalization of CB1 receptors in GABAergic afferents to the substantia nigra pars reticulata (SNr), whereas dopaminergic cells are restricted to the pars compacta (SNc). The segregation of CB1 receptors and catecholaminergic transmission is also observed in the hippocampus-dentate gyrus (Hpc-DG, panel G).

Enzymes. Fatty acid amide hydrolase is present in large principal neurons, such as the pyramidal cells of the cerebral cortex, the pyramidal cells of the hippocampus, the Purkinje cells of the cerebellar cortex and the mitral cells of the olfactory bulb.

Immunocytochemical analysis of these brain regions revealed a complementary pattern of FAAH and CB1 expression with CB1 immunoreactivity occurring in fibres surrounding FAAH-immunoreactive cell bodies and/or dendrites (Egertova et al., 2003). This complementary distribution suggests that FAAH closely controls the duration of cannabinoid effects, although there are sites where this association does not occur, such as the outflow nuclei of basal ganglia. Monoglyceride lipase is located mainly in the hippocampus, cortex, cerebellum and anterior thalamus, with moderate expression in the extended amygdala, including the shell of the nucleus accumbens (Dinh et al., 2002). Comparison of the distribution of FAAH and MAGL at the cellular level shows that FAAH is primarily a postsynaptic enzyme, whereas MAGL is presynaptic. The spatial segregation of the two enzymes suggests that anandamide and 2-AG signalling may subserve functional roles that also involve spatial segregation, raising a controversy with respect to the nature and function of the retrograde endocannabinoid signal (Gulyas et al., 2004).

Transporter. The distribution of the anandamide transporter has been only partially characterized because the transporter has not been cloned. The distribution of transport activity is highest in areas expressing CB1 receptors, such as the hippocampus, the amygdala, the striatum and the somatosensory, motor and limbic areas of the cortex. Transport activity is also present in areas with low expression of the CB1 receptor, such as the thalamus and the hypothalamus (Beltramo et al., 1997; Giuffrida et al., 2001).

Pharmacology of the endogenous cannabinoid system

During the last twenty years, and especially after the discovery of the CB1 receptor and anandamide, an intense research effort has yielded numerous series of drugs that interact with most of the main elements of the endogenous cannabinoid system. Today we have drugs that bind to the CB1 receptor as agonists or antagonists, drugs that block the endocannabinoid transport and drugs that inhibit the activity of FAAH. We lack specific NAT, PLD, sn1-DAGL and MAGL inhibitors. Both in vitro and in vivo bioassays have been used to evaluate the activity of the new compounds. Prior to the availability of radioligand cannabinoid receptors, in vitro assays included the inhibition of forskolin-stimulated cAMP production and the inhibition of electrically evoked contractions of isolated smooth muscle preparations. Smooth muscle preparations most often used for the bioassay of cannabinoids are the mouse-isolated vas deferens and the myenteric plexus-longitudinal muscle preparation from the guinea pig small intestine. These bioassays, which are particularly sensitive, rely on the ability of cannabinoid

receptor agonists to act via the CB1 receptors to inhibit electrically evoked contractions. In vivo bioassays include behavioural tests for analgesia and locomotion. A cluster of four effects (analgesia, hypothermia, immobility and catalepsy) in mice constituting the 'mouse tetrad', is classically considered as a signature of cannabimimetic activity. The recent availability of mouse knockouts for the cannabinoid receptors and FAAH (Ledent et al., 1999; Cravatt et al., 2001) has facilitated these studies, offering a reliable model in the search for selective compounds.

What is the logic of a cannabinoid approach to pharmacotherapeutics? Cannabinoid receptor agonists may be designed to mimic the signalling processes mediated by anandamide and 2-AG, mainly in pathological situations where a boost in cannabinoid receptor stimulation might be needed. Cannabinoid receptor antagonism might be the approach selected in conditions with enhanced endocannabinoid signalling. Transport inhibition and inhibition of degradation are more sophisticated approaches, both oriented towards magnifying the tonic actions of endocannabinoids. A rational use of these therapeutic strategies requires the identification and evaluation of the functional status of endocannabinoid signalling in reference disorders. Thus, a deficit of anandamide signalling during conditions of stress might be counteracted by the blockade of anandamide degradation (Kathuria et al., 2003).

As a summary of cannabinoid pharmacology, Table 2 shows the reference compound for each molecular target, indicating Ki in the case of ligand–receptor interaction or IC50 in the case of enzymatic inhibitors.

Table 2.Targeting the endogenous cannabinoid system: synthetic drugs of reference for cannabinoid CB1 and CB2 receptors, anandamide transporter (AT) and endocannabinoid degradation enzyme, fatty acid amidohydrolase (FAAH)

Name	Target	Action	Ki/IC50 (nM)	Reference
ACEA	CB1	Agonist	1.4	Hillard et al., 1999
SR141716A	CB1	Antagonist	5.6	Rinaldi-Carmona et al., 1994
HU-308	CB2	Agonist	22.7	Hanus et al., 1999
SR 144528	CB2	Antagonist	0.60	Rinaldi-Carmona et al., 1998
UCM 707	AT	Blocker	800	Lopez-Rodriguez et al., 2001
OL-135	FAAH	Inhibitor (reversible)	2.1	Lichtman et al., 2004
URB 597	FAAH	Inhibitor (irreversible)	4.6	Kathuria et al., 2003

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Cannabinoid receptor agonists. According to the International Union of Pharmacology (reviewed in Howlett et al., 2002), cannabinoid agonists can be divided into classical cannabinoids, non-classical cannabinoids, aminoalkylindoles and eicosanoids. New series of compounds have been recently described, including diarylether sulfonylesters (Mauler et al., 2002) and pyrrole derivatives (Tarzia et al., 2003b).

Classical cannabinoids are tricyclic dibenzopyran derivatives that are either compounds occurring naturally in the plant C. sativa, or synthetic analogues of these compounds. The most representative forms are Δ9-THC (Fig. 1), a partial agonist at both the CB1 and CB2 receptors and the main psychoactive constituent of Cannabis, along with 11-hydroxy-Δ8-THC-dimethylheptyl (HU-210), a synthetic compound that displays the highest potency at the CB1 receptor (Howlett et al., 2002). Classical cannabinoids are usually CB1/CB2 agonists, although changes in the THC molecule have led to the synthesis of selective CB2 receptor agonists such as HU-308 (Hanus et al., 1999).

Non-classical cannabinoids are synthetic THC analogues that lack the dihydropyran ring. The most representative form is the Pfizer compound CP-55 940, a potent and complete agonist at both the CB1 and CB2 receptors, which was used to characterize the CB1 receptor for the first time (Devane et al., 1988; Herkenham et al., 1991).

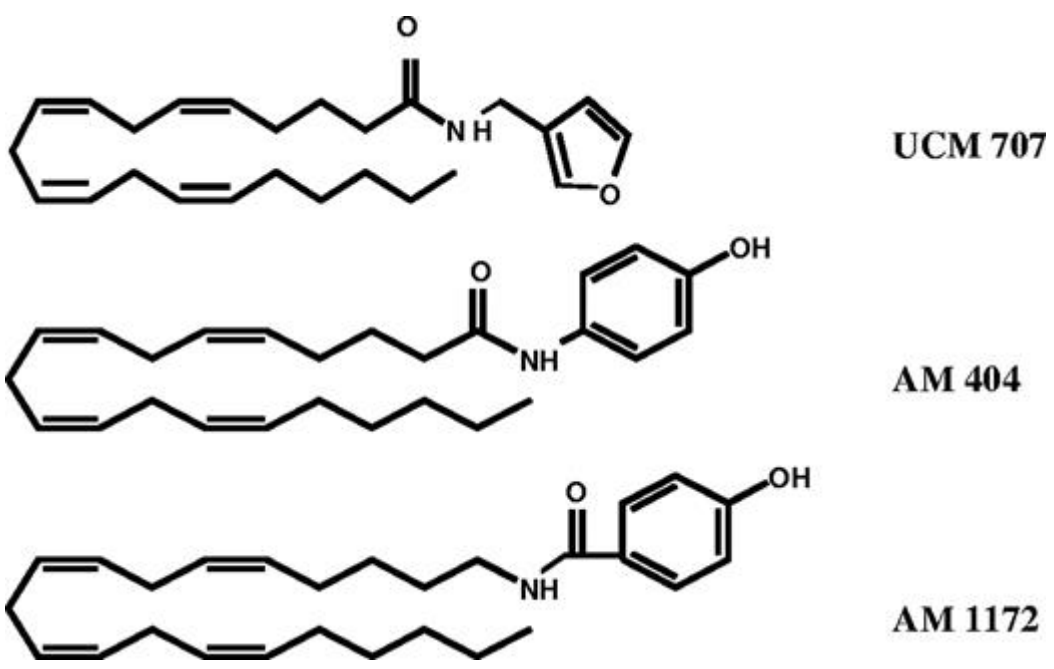
Aminoalkylindoles were the first non-cannabinoid molecules that displayed cannabimimetic activity (Pacheco et al., 1991). R-(+)-WIN-55,212–2 (Fig. 1) is the most representative form, and it behaves as a complete agonist at both the CB1 and CB2 receptors, with higher intrinsic activity at the CB2 receptor.

Eicosanoids are the prototypic endocannabinoids (Fig. 1), of which anandamide (a partial agonist at both the cannabinoid receptors) and 2-AG (a complete agonist at both the CB1 and CB2 receptors) are the most representative compounds. Based on the structure of anandamide, minor chemical changes have led to the development of the first generation of CB1-selective agonists, of which R(+)-methanandamide and arachidonyl-2′-chloroethylamide (ACEA) (Table 2) are the most representative forms (Hillard forms et al., 1999).

Cannabinoid receptor antagonists. Several series of compounds have been developed as CB1 receptor antagonists. The most representative

are diarylpyrazoles, substituted benzofuranes, aminoalkylindoles and triazole derivatives.
Diarylpyrazoles include both the first CB1 receptor antagonist synthesized (SR 141716A, Rinaldi-Carmona et al., 1994) and the first CB2 receptor antagonist (SR 144528). They were synthesized by Sanofi and are considered the reference antagonists. However, they are not neutral antagonists since they display significant inverse agonist properties. Modification of the SR 141716A molecule has yielded other CB1 receptor antagonists with improved properties, including SR 147778 and AM 281 (Howlett et al., 2002; Rinaldi-Carmona et al., 2004). Diarylpyrazoles are orally active and are currently under clinical trials for the treatment of obesity.
Substituted benzofuranes include LY 320135, a CB1 receptor antagonist with affinity at serotonin and muscarinic receptors (Felder et al., 1998).
Aminoalkylindoles include a CB2 receptor antagonist, AM 630, which also displays activity as a low-affinity partial CB1 agonist (Howlett et al., 2002).
Triazole derivatives include LH-21 (Jagerovic et al., 2004), an in vivo CB1 antagonist with a paradoxic low affinity in vitro for CB1 receptors and devoid of inverse agonist properties.
Uptake blockers. Based on the structure of anandamide, a series of eicosanoid derivatives that have the ability to block anandamide transport have been synthesized. The molecular structures of the three prototypical uptake blockers are depicted in Fig. 4. The first and best studied transport inhibitor is AM 404 (Beltramo et al., 1997). The administration of AM 404 results in the accumulation of anandamide and potentiates the effects of exogenously administered anandamide. The compound AM 404 can be degraded by FAAH and behaves as an agonist of vanilloid receptors. A second series of compounds is represented by UCM 707, which displays a higher affinity at the transporter than AM 404 (Lopez-Rodraíguez et al., 2001; De Lago et al., 2002). A latest addition is AM 1172, a FAAH-resistant transport inhibitor that allows the study of anandamide uptake processes without interference in FAAH activity (Fegley et al., 2004). However the IC50 of AM 1172 (2000 nM) is lower than that reported for UCM 707 (800 nM).

Fig. 4.



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Structure of three anandamide uptake blockers. UCM 707 is the compound with the highest affinity at the anandamide transporter. AM 404 was the first blocker designed and has been extensively described. Both molecules, however, had a significant impact on the activity of the fatty acid amidohydrolase (FAAH), the enzyme that degrades anandamide. AM 1172 is a recently described compound without inhibitory action at FAAH, which has been used to demonstrate the independence of anandamide transport and degradation processes.
Inhibitors of fatty acid amide hydrolase. As in the case of the cannabinoid receptors, different lines of research have led to the discovery of chemically heterogeneous FAAH inhibitors. The earlier inhibitors described consisted of reversible electrophilic carbonyl inhibitors (trifluoromethyl ketones, alpha-keto esters and amides, and aldehydes) or irreversible inhibitors (sulfonyl fluorides and fluorophosphonates) incorporated into the fatty acid structures. Based on the structure of alpha-trifluoromethyl ketones a series of potent inhibitors were developed. Of these, alpha-keto N4-oxazolopyridine provides inhibitors that are 102–103 times more potent than the corresponding trifluoromethyl ketones (Boger et al., 2000). A recent series of alpha heterocycles has been shown to possess very high potency and selectivity to reversibly inhibit FAAH activity in vivo and in vitro. The most potent of these new compounds is OL-135, which exhibits IC50 in the low nanomolar range (Lichtman et al., 2004). A different strategy has been selected by the group of Piomelli et al., who have developed exceptionally potent irreversible FAAH inhibitors, which exhibit a promising anxiolytic profile (Kathuria et al., 2003; Tarzia et al., 2003a). These new classes of inhibitors are carbamate derivatives capable of directly interacting with the serine nucleophile of FAAH. However, these new inhibitors, although extremely potent, are not selective because they may potentially inactivate other serine hydrolases such as heart triacylglycerol hydrolase (Lichtman et al., 2004).

Physiology of the endogenous cannabinoid system

The ubiquitous presence of the endogenous cannabinoid system correlates with its role as a modulator of multiple physiological processes. A comprehensive analysis of all the functions of the endocannabinoids is beyond the scope of the present review. The reader will find an extensive list of recent reviews that explore the physiological relevance of the endogenous cannabinoid system, as depicted in Table 1. In this section, we focus on the cellular and system physiological events mediated by endocannabinoids that are relevant to our understanding of the contribution of the endogenous cannabinoid system in alcoholism.
Cellular physiology. As described in the section on biochemistry of the endogenous cannabinoid system, endocannabinoids are released upon demand after cellular depolarization or receptor stimulation in a calcium-dependent manner. Once produced, they act on the

cannabinoid receptors located in the cells surrounding the site of production. This property indicates that endocannabinoids are local mediators similar to the autacoids (e.g. prostaglandins). In the CNS, the highly organized distribution of endocannabinoid signalling elements in GABAergic and glutamatergic synapses and their preservation throughout evolution suggests a pivotal role in synaptic transmission. Because of the inhibitory effects on adenlyl cyclase, the activation of K+ currents and the inhibition of Ca2+ entry into cells, the net effect of the CB1 receptor stimulation is a local hyperpolarization that leads to the general inhibitory effects described. If endocannabinoids act postsynaptically they will counteract the activatory inputs entering the postsynaptic cells. This mechanism has been proposed for postsynaptic interactions with dopaminergic transmission (Felder et al., 1998; Rodríguez de Fonseca et al., 1998; Giuffrida et al., 1999). Despite its importance, this effect is secondary to the important presynaptic actions whose existence is supported by two facts: (i) the concentration of the CB1 receptors in presynaptic terminals and (ii) the well-documented inhibitory effects of the CB1 receptor agonists on the release of GABA, glutamate, acetylcholine and noradrenaline (Schlicker and Kathmann, 2001; Piomelli, 2003). This inhibitory effect has been demonstrated for neuropeptides such as corticotrophin-releasing factor and cholecystokinin as well (Rodríguez de Fonseca et al., 1997; Beinfeld and Connolly, 2001). Presynaptic inhibition of neurotransmitter release is associated with the inhibitory action of endocannabinoids on Ca2+ presynaptic calcium channels via the activation of CB1 receptors. Presynaptic inhibition of transmitter release by endocannabinoids may adopt two different forms of short-term synaptic plasticity, depending on the involvement of GABA or glutamate transmission, respectively: depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE) (Wilson and Nicoll, 2002; Diana and Marty, 2004). Both forms of synaptic plasticity involve the initial activation of a postsynaptic large projecting neuron (pyramidal or Purkinje cells) that sends a retrograde messenger to a presynaptic GABA terminal (DSI) or a presynaptic glutamate terminal (DSE), inducing a transient suppression of either the presynaptic inhibitory or the presynaptic excitatory input. The contribution of endocannabinoids to these forms of short-term synaptic plasticity has been described in the hippocampus (Wilson and Nicoll, 2001; Wilson et al., 2001) and the cerebellum (Diana et al., 2002). The nature of the endocannabinoid system acting as a retrograde messenger is still unknown. The role of endocannabinoid-induced DSI or DSE seems to be the coordination of neural networks within the hippocampus and the cerebellum that are involved in relevant physiological processes, such as memory or motor coordination.

Additional forms of endocannabinoid modulation of synaptic transmission involve the induction of long-term synaptic plasticity, namely long-term potentiation (LTP) and long-term depression (LTD). Both forms of synaptic plasticity involve long-term changes in the efficacy of synaptic transmission in glutamatergic neurons, which have a major impact on consolidation and remodelling of the synapsis. Activation of the cannabinoid receptors prevents the induction of LTP in the hippocampal synapses (Stella et al., 1997) and a facilitation of LTD in the striatum (Gerdeman et al., 2002) and the nucleus accumbens (Robbe et al., 2002). In the hippocampus, the endocannabinoid messengers regulate a form of LTD that affects inhibitory GABAergic neurons (Chevaleyre and Castillo, 2003).

Overall, endocannabinoids act as local messengers that adjust synaptic weight and contribute significantly to the elimination of information flow through specific synapses in a wide range of time frames. The fact that cannabinoid receptor stimulation has a major impact on second messengers involved not only in synaptic remodelling (Derkinderen et al., 1996; Piomelli, 2003) but also in neuronal differentiation (Rueda et al., 2002) and neuronal survival (Panikashvili et al., 2001; Marsicano et al., 2003) indicates that the signalling system is a major homeostatic mechanism that guarantees a fine adjustment of information processing in the brain and provides counterregulatory mechanisms aimed at preserving the structure and function of major brain circuits. Both processes are relevant for homeostatic behaviour such as motivated behaviour (feeding, reproduction, relaxation, sleep) and emotions, as well as for cognition, since learning and memory require dynamic functional and morphologic changes in brain circuits. An experimental confirmation of this hypothetical role of the endogenous cannabinoid system was the demonstration of its role in the control of the extinction of aversive memories (Marsicano et al., 2002; Terranova et al., 1996).

System physiology. The cellular effects of endogenous cannabinoids have a profound impact on the main physiological systems that control body functions (Table 1). Despite the peripheral modulation of the immune system, vascular beds, reproductive organs, gastrointestinal motility and metabolism, the endogenous cannabinoid system tightly regulates perception processes including nociception (cannabinoids are potent analgetics, Martin and Litchman, 1998) and visual processing in the retina (Straiker et al., 1999). Additional functions exerted by the endogenous cannabinoid system involve the regulation of basal ganglia and cerebellar circuits, where it is involved in the modulation of implicit learning of motor routines (Rodriguez de Fonseca et al., 1998).

Among the varied functions in which the endogenous cannabinoid system is engaged, the homeostatic control of emotions and the regulation of motivated behaviour merit special attention because of its impact on human diseases, including addiction. The endogenous cannabinoid system controls the motivation for appetite stimuli, including food and drugs (Di Marzo et al., 1998, 2001; Navarro et al., 2001; Gomez et al., 2002). The positive effects of endocannabinoids on motivation seem to be mediated not only by the peripheral sensory systems in which cannabinoid receptors are present (i.e. the promotion of feeding induced by cannabinoid CB1 receptor agonists, Gomez et al., 2002), but also by the action of endocannabinoids on the reward system, a set of in-series circuits that link the brain stem, the extended amygdala and the frontal executive cortex. The endogenous cannabinoid system is widely distributed in the extended amygdala, a set of telencephalic nuclei located in medial septal neurons, the nucleus accumbens shell and amygdalar complex, and are involved in the control of motivated behaviour, conditioned responses and gating-associated emotional responses. This hypothesis is supported by two facts: the inhibition of motivated behaviour observed after administration of a cannabinoid antagonist (Colombo et al., 1998; Navarro et al., 2001) and the reward deficits observed in the CB1 receptor knockout mice (Ledent et al., 1999; Maldonado and Rodríguez de Fonseca, 2002; Sanchis-Segura et al., 2004). Research on the neurobiological basis of endocannabinoid effects on motivated behaviour has focused on endocannabinoid–dopamine interaction as well as on the role of the endocannabinoid system in habit learning and conditioning. The extended amygdala is the target of the ascending mesocorticolimbic projections of the ventral tegmental area (VTA) dopaminergic neurons, a subset of mesencephalic neurons that display a consistent response to drugs of major abuse, which appear to be a common substrate for the reward properties of drugs of dependence (Maldonado and Rodríguez de Fonseca, 2002). Most drugs of dependence activate the VTA

dopaminergic neurons, as monitored by the dopamine release in terminal areas, especially in the nucleus accumbens and prefrontal cortex, or by the firing rates of VTA dopaminergic neurons. THC and other CB1 receptor agonists increase dopamine efflux in the nucleus accumbens and prefrontal cortex and increase the dopaminergic cell firing in the VTA (for review see Gardner and Vorel, 1998). This effect is not caused by the direct activation of dopaminergic neurons because they do not express CB1 receptors (Julian et al., 2003). Although the effects of cannabinoid agonists on dopamine release in the projecting areas (i.e. nucleus accumbens) can be blocked by the opioid antagonist naloxone, the increase in VTA dopaminergic cell firing cannot be blocked. This discrepancy may suggest the existence of a differential role for endogenous opioid systems as the modulators of cannabinoid actions in dopamine cell bodies with respect to their axon terminals. Cannabinoid effects might also involve glutamatergic and GABAergic inputs to the nucleus accumbens and VTA, because presynaptic CB1 receptors regulate glutamate and GABA release in these areas, inducing LTD (Schlicker and Kathmann, 2001; Robbe et al., 2002). In agreement with these actions of cannabinoids in brain reward circuits, repeated cannabinoid exposure can induce behavioural sensitization similar to that produced by other drugs of dependence. Chronic cannabinoid administration also produces cross-sensitization to the locomotor effects of psychostimulants (Maldonado and Rodríguez de Fonseca, 2002). Because endocannabinoids induce LTD in the nucleus accumbens (which affect glutamatergic inputs coming from the prefrontal cortex), they probably regulate the acquisition of habit learning and conditioned responses relevant to the progressive loss of control that characterize drug addiction (Maldonado and Rodríguez de Fonseca, 2002). Interestingly, administration of a CB1 receptor antagonist blocks cue-induced reinstatement to heroin and cocaine self-administration (De Vries et al., 2001, 2003). The importance of the endogenous cannabinoid system in the control of motivated behaviour goes far beyond the control of processing ongoing reward signals. The CB1 receptors are apparently involved in the control of reward homeostasis (Sanchis-Segura et al., 2004). Moreover, when cannabinoid homeostatic mechanisms are not adequate to restore the lost equilibrium in reward control derived from continuous uncontrolled exposure to a reinforcer (e.g. opiates or alcohol), allostatic changes involving CB1 receptors are set in motion to counteract the spiralling distress imposed on the reward circuit. This has been demonstrated in rodents exposed to cycles of dependence–abstinence to alcohol and morphine (Navarro et al., 2001; Rimondini et al., 2002). In this model, a history of dependence is associated with a permanent upregulation of the expression of CB1 receptors in reward-related areas and with an enhanced sensitivity to reward disruption induced by cannabinoid receptor antagonists (Rodríguez de Fonseca et al., 1999; Rimondini et al., 2002). Whether these allostatic changes occur in other models of motivated behaviour (i.e. feeding) remains to be determined.

Cannabinoid receptors are not only associated with motivational disturbances, but also related to emotional processing. A key station for the endocannabinoid regulation of emotions is the amygdalar complex. Endocannabinoids are able to depress the release of glutamate and corticotropin-releasing factor, reducing the amygdalar output and the activity of basolateral inhibitory GABA projections to the central nucleus of the amygdala, thereby activating the amygdalofugal pathway (Rodríguez de Fonseca et al., 1996, 1997; Navarro et al., 1997; Marsicano et al., 2002; Piomelli, 2003). The final balance will lead to anxiety or anxiolysis, depending on the rate of activation of descending projections of the central nucleus of the amygdala to the hypothalamus (endocrine responses) and brain stem (behavioural and autonomic responses). However, recent studies indicate that anxiolysis is the normal response to enhanced cannabinoid transmission in the limbic system, as reflected by the phenotype of FAAH knockout mice and the effects of FAAH inhibitors (Cravatt et al., 2003; Kathuria et al., 2003). The induction of anxiety by cannabinoid receptor antagonists (Navarro et al., 1997) supports this notion as well.

A practical approach: role for the endocannabinoid system in alcoholism

The presence of the endogenous cannabinoid system in reward circuits and its role in motivational and emotional homeostasis suggests that drugs which modulate cannabinoid signalling might serve as therapeutic tools in drug addiction. In accordance with this rationale, the CB1 receptor antagonists are able to modulate opioid self-administration in rodents (Navarro et al., 2001). Extending this hypothesis, converging research lines have established a role for both anandamide and the CB1 receptor in alcohol dependence (Hungund and Basaravajappa, 2000; Hungund et al., 2002; Mechoulam and Parker, 2003). The administration of CB1 receptor agonists promotes alcohol intake (Colombo et al., 2002), whereas the administration of a CB1 receptor antagonist decreases alcohol self-administration, especially in animals with a history of alcohol dependence (Rodríguez de Fonseca et al., 1999) or in alcohol-preferring rat lines (Colombo et al., 1998). Molecular studies have shown that chronic alcohol administration is associated with an increased formation of both anandamide and its membrane precursor NAPE (Basavarajappa and Hungund, 1999). Chronic alcohol exposure also resulted in the stimulation of a second endocannabinoid, 2-AG (Basavarajappa et al., 2000). Animal studies also revealed that chronic exposure to alcohol downregulated the CB1 receptors in the brain (Basavarajappa et al., 1998). Finally, a recent gene screening study has identified the CB1 receptor as one of the genes whose expression is permanently affected by serial cycles of alcohol dependence and withdrawal (Rimondini et al., 2002). These data indicate a role for the endogenous cannabinoid system as a relevant contributor to alcoholism. Human gene studies support this experimental hypothesis, since a linkage between clinical forms of alcoholism and polymorphisms and/or mutations of the genes encoding either the CB1 receptor (Comings et al., 1997; Schmidt et al., 2002) or the FAAH (Sipe et al., 2002), the enzyme responsible for AEA inactivation (Cravatt et al., 1996), have been described. In the present issue, the reader will find additional experimental approaches to the role of the endogenous cannabinoid system in alcoholism.

CONCLUSION

Since the discovery of anandamide, the increasing information on the physiological roles played by the endogenous cannabinoid system and its contribution to pathology have led to this signalling system becoming more important in neurobiology. The intense pharmacological research based on this information has yielded, in a very short time, potent, selective drugs targeting the endogenous cannabinoid system that have opened up new avenues for the understanding and treatment of major diseases including cancer, pain, neurodegeneration, anxiety and addiction. This is a very promising starting point for a new age that takes over from the ancient use of Cannabis as a medicine. Now is the time for clinical trials aimed at evaluating the efficacy of cannabinoid drugs in disorders lacking effective therapeutic approaches, such as alcoholism.

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Cocaine-Induced Endocannabinoid Mobilization in the Ventral Tegmental Area
https://www.sciencedirect.com/science/article/pii/S2211124715009262

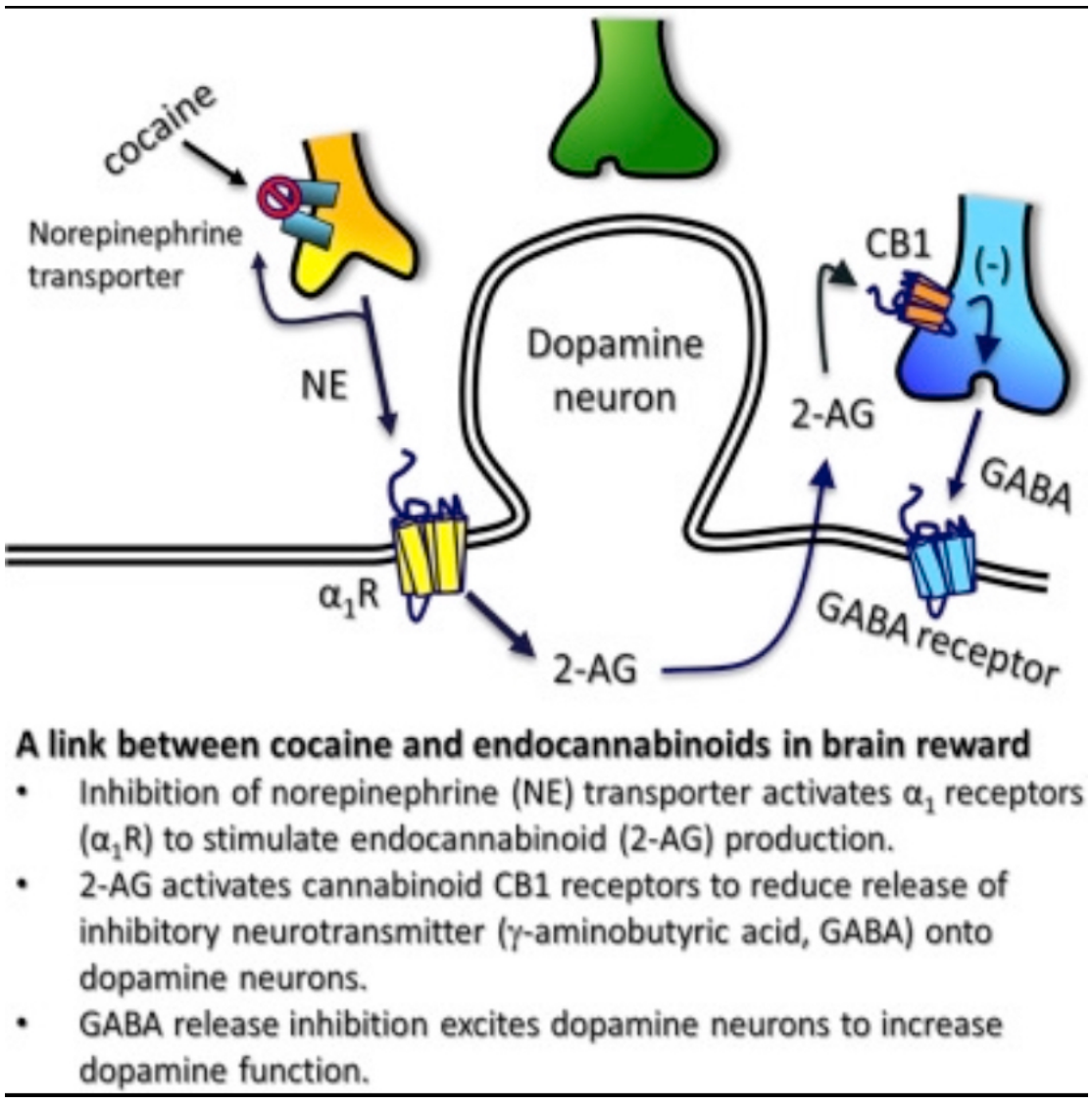
Highlights

- Endogenous cannabinoids modulate the behavioral effects of cocaine
- Cocaine mobilizes 2-arachidonoylglycerol (2-AG) in the midbrain
- Cocaine-mobilized 2-AG disinhibits dopamine (DA) neurons and increases DA release
- Cocaine mobilizes 2-AG via Gq/11-coupled receptor coupling to internal calcium

Summary

Cocaine is a highly addictive drug that acts upon the brain's reward circuitry via the inhibition of monoamine uptake. Endogenous cannabinoids(eCB) are lipid molecules released from midbrain dopamine (DA) neurons that modulate cocaine's effects through poorly understood mechanisms. We find that cocaine stimulates release of the eCB, 2-arachidonoylglycerol (2-AG), in the rat ventral midbrain to suppress GABAergic inhibition of DA neurons, through activation of presynaptic cannabinoid CB1 receptors. Cocaine mobilizes 2-AG via inhibition of norepinephrine uptake and promotion of a cooperative interaction between Gq/11-coupled type-1 metabotropic glutamate and α1-adrenergic receptors to stimulate internal calcium stores and activate phospholipase C. The disinhibition of DA neurons by cocaine-mobilized 2-AG is also functionally relevant because it augments DA release in the nucleus accumbensin vivo. Our results identify a mechanism through which the eCB system can regulate the rewarding and addictive properties of cocaine.

Graphical Abstract


cocaine
Norepinephrine transporter
NE
Dopamine neuron
CB1
(-)
2-AG
GABA
α_1R
2-AG
GABA receptor
A link between cocaine and endocannabinoids in brain reward
• Inhibition of norepinephrine (NE) transporter activates α_1 receptors (α_1R) to stimulate endocannabinoid (2-AG) production.
• 2-AG activates cannabinoid CB1 receptors to reduce release of inhibitory neurotransmitter (γ-aminobutyric acid, GABA) onto dopamine neurons.
• GABA release inhibition excites dopamine neurons to increase dopamine function.

Introduction

The ventral tegmental area (VTA) is a central component of a mammalian brain reward circuit involved in processing environmental information to ensure survival. The VTA contains neurons that project extensively to the forebrain and release dopamine (DA). These neurons mediate the rewarding properties of all abused drugs (
Di Chiara and Imperato, 1988
,
Wise and Rompre, 1989
) and shift their activity from pacemaker to burst firing during exposure to drugs or stimuli predicting drug availability (
Cooper, 2002

,
Covey et al., 2014
). This enhancement of DA neuron activity results in increased DA in projection sites, such as the nucleus accumbens(NAc), which is critical to drug reinforcement (
Di Chiara and Bassareo, 2007
,
Phillips et al., 2003
).
Cannabinoid CB1 receptors (CB1R) are coupled to G-proteins, are extensively expressed on axon terminals in the CNS, and inhibit release of excitatory and inhibitory neurotransmitters to sculpt postsynaptic activity (
Alger and Kim, 2011
,
Kano et al., 2009
). CB1Rs are also activated by the primary psychoactive constituent of cannabis, Δ9-tetrahydrocannabinol (Δ9-THC), to mediate the physiological and psychotropic effects of this drug in humans (
Hoffman and Lupica, 2013
). Molecules, known as endogenous cannabinoids(eCBs), derived from membrane phospholipids, also activate CB1Rs to mediate short- and long-term forms of synaptic plasticity (
Kano et al., 2009
). Anandamide (
Devane et al., 1992
) and 2-arachidonoylglycerol (2-AG) (
Mechoulam et al., 1995
) are the best characterized eCBs, with 2-AG most closely associated with activity-dependent modulation of synapses (
Kano et al., 2009
). 2-AG can be mobilized during transient postsynaptic increases in Ca2+ through activation of voltage-dependent Ca2+ channels (
Kreitzer and Regehr, 2001
,
Maejima et al., 2001
,
Ohno-Shosaku et al., 2001
,
Wilson and Nicoll, 2001
). This type of 2-AG release mediates depolarization-induced suppression of inhibition (DSI) or excitation (DSE), in which 2-AG from postsynaptic cells acts to retrogradely activate CB1Rs on axon terminals to inhibit GABA or glutamate release, respectively. 2-AG synthesis can also be triggered through activation of Gαq/11-coupled metabotropic receptors via their coupling to phospholipase C-β (PLCβ). PLCβ hydrolyzesphosphatidylinositol 4,5-bisphosphate to form 1,2-dacylglycerol (DAG) and inositol triphosphate. DAG is then hydrolyzed by diacylglycerol lipase-α (DGLα) to form 2-AG (
Hashimotodani et al., 2005
,
Kano et al., 2009
,
Maejima et al., 2005
). Receptor-mediated 2-AG release can also be strongly augmented by coincident elevation of intracellular Ca2+ (
Hashimotodani et al., 2007
,
Kano et al., 2009
,
Maejima et al., 2005
).
eCBs are released from VTA DA neurons during depolarization (
Melis et al., 2004
) or conditions promoting burst activity (
Riegel and Lupica, 2004
). Furthermore, DGLα is strategically located on postsynaptic membranes adjacent to presynaptic CB1Rs in the VTA (
Mátyás et al., 2008
). All abused drugs, including Δ9-THC, increase DA release in forebrain areas such as the NAc. The cannabinoid-induced increase in DA likely occurs through an increase in VTA DA neuron firing rate and bursting (
French et al., 1997
,

Gessa et al., 1998
). Since cannabinoids act at CB1Rs in the VTA to inhibit the release of the inhibitory neurotransmitter GABA (
Riegel and Lupica, 2004
,
Szabo et al., 2002
), we hypothesize that disinhibition of VTA DA neurons leads to increases in their activity and phasic release of DA in the NAc (
Cheer et al., 2007
,
Lupica et al., 2004
,
Riegel and Lupica, 2004
).
The eCB system also regulates the rewarding actions of many abused drugs (
Lupica and Riegel, 2005
). Antagonism of CB1Rs reduces drug-induced reward-related behavior and the associated elevation of NAc DA (
Cheer et al., 2007
,
Li et al., 2009
). The rewarding property of the psychostimulant cocaine is primarily attributed to its ability to increase forebrain DA levels via inhibition of the DA transporter (DAT) (
Ritz et al., 1987
,
Thomsen et al., 2009
). However, because cocaine-induced elevations of DA release events depend on VTA DA neuron firing (
Sombers et al., 2009
) and eCB signaling (
Cheer et al., 2007
), eCB release in the VTA may be critical to cocaine reinforcement.
Here, we find that cocaine mobilizes 2-AG, which then activates CB1Rs to inhibit GABA release onto DA neurons, and that cocaine's ability to elevate NAc DA in awake rats is attenuated by disruption of 2-AG signaling. Mobilization of 2-AG by cocaine depends on activation of Gq/11-coupled receptors, initiation of Ca2+ oscillations, and activation of PLC. We propose that cocaine increases DA in the NAc and reinforces behavior, in part, through disinhibition of DA neurons via suppression of GABA release by cocaine-induced 2-AG mobilization.

Results

Inhibition of GABA Release onto VTA DA Neurons by 2-AG

IPSCs mediated by GABAB receptors on VTA DA neurons (S1a) are inhibited by presynaptic CB1Rs (
Riegel and Lupica, 2004
). Here, the synthetic CB1Ragonist WIN55,212-2 (1 μM) inhibited GABAB IPSCs (77.9% ± 3.9% of control, n = 5; $p < 0.05$, paired t test) (
Figures 1
A and 1B). These responses were also increased by the CB1R antagonist/inverse agonist, AM251 (2 μM; 115.4% ± 1.6% of control, n = 6; $p < 0.01$, paired t test), suggesting that eCBs tonically inhibit GABAB IPSCs. Because many CB1R antagonists, such as AM251, are also inverse agonists (
Landsman et al., 1997
), their reversal of eCB effects may instead reflect allosteric modulation of CB1Rs. However, a "neutral" CB1R antagonist, 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-[(E)-piperidinoiminomethyl]-1H-pyrazole (PIMSR1; 2 μM) (
Hurst et al., 2006
) also increased GABABIPSCs in putative DA neurons (121.3% ± 5.7% of control, n = 6; $p < 0.05$, paired t test). Moreover, this did not differ significantly from that produced by AM251 ($p = 0.35$, unpaired t test,
Figures 1
A and 1B). This suggests that AM251 and PIMSR1 increased GABAB IPSC amplitude through antagonism of tonically released eCB acting at CB1Rs and not through allosteric modulation. As the effect of AM251 did not differ from PIMSR1, it was used in subsequent experiments.

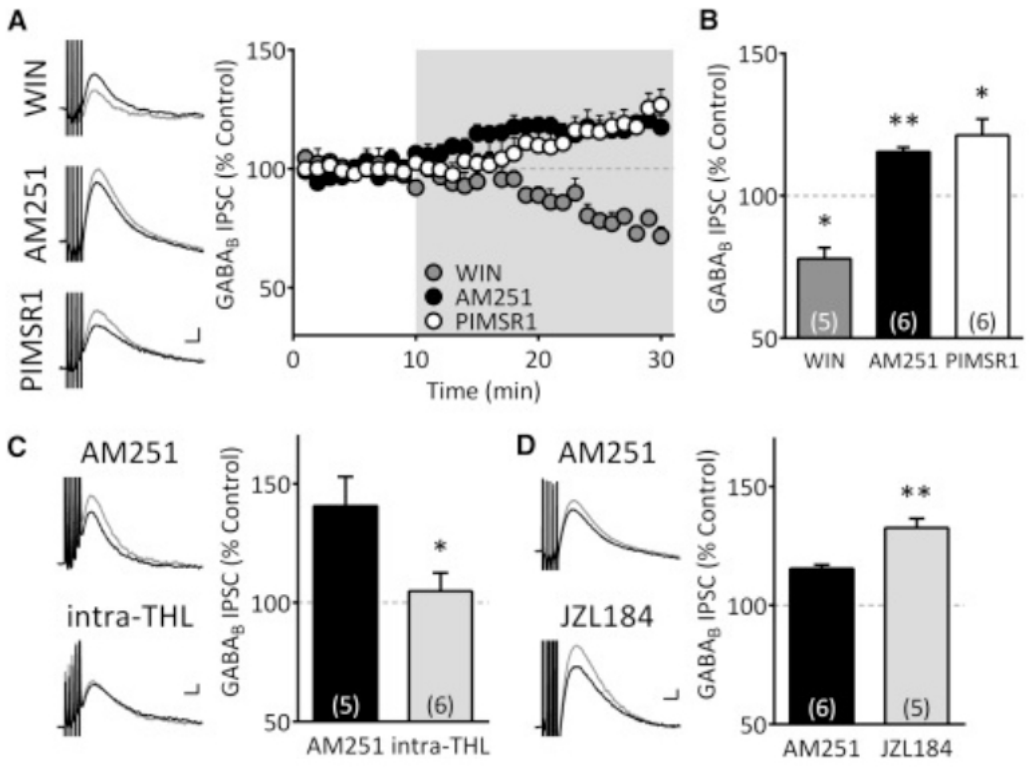


Figure 1. Tonic Inhibition of GABA Signaling in VTA DA Neurons Is Mediated by 2-AG

(A) Sample traces (left) and time-courses of GABABIPSCs (right) during CB1R ligand treatment; CB1R agonist WIN55,212-2 (WIN, 1 μM), CB1R antagonist/inverse agonist, AM251 (2 μM), and neutral antagonist PIMSR1 (2 μM). Gray traces in all figures indicate those during treatment. Gray area in time course indicates period of drug application. Scale bar, 100 ms, 10 pA.

(B) Summary of CB1R ligand effects on GABAB IPSCs ($F(2,14) = 30.75$, $p < 0.0001$, one-way ANOVA, $*p < 0.01$, $**p < 0.001$, Tukey's post hoc).

(C) The AM251-mediated increase in GABAB IPSCs is prevented by inclusion of the DGL inhibitor, THL (5 μM), in the pipette ($*p < 0.05$, unpaired t test). Scale bar, 100 ms, 10 pA.

(D) The AM251-induced increase of GABAB IPSCs is enhanced by the MAG lipase inhibitor JZL184(10 μM; $**p < 0.01$, unpaired t test). Scale bar, 100 ms, 10 pA.

We next determined which eCB exerted control over GABAB IPSCs in putative VTA DA neurons. 2-AG is derived from membrane phospholipids via the sequential activity of PLC and DGLα (
Kano et al., 2009
). 2-AG is then inactivated by the enzyme monoacylglycerol lipase (MGL) in presynaptic terminals. Inclusion of the DGLαinhibitor tetrahdydrolipstatin (THL, 5 μM) in the intra-pipette solution prevented the enhancement of GABAB IPSCs by AM251 (AM251 = 140.7% ± 12.3%, n = 5; AM251 + intra-THL = 104.9% ± 7.7%, n = 6; unpaired t test, $p < 0.05$;
Figure 1
C). In contrast, the increase in GABAB IPSCs produced by AM251 was significantly larger in the presence of the MGL inhibitor JZL184 (10 μM; AM251 alone = 115.4% ± 1.6% of control, n = 6; AM251 + JZL184 = 132.6% ± 3.9% of control, n = 5; unpaired t test, $p < 0.01$;
Figure 1
D). This demonstrates that 2-AG is tonically released from putative DA neurons to inhibit GABABIPSCs via activation of CB1Rs.

Cocaine Increases DA Transients in the NAc via eCB Action in the VTA

Systemic cocaine administration increases DA transient signals measured by fast scan cyclic voltammetry (FSCV) in the NAc and this is attenuated by systemic administration of the CB1R antagonist, rimonabant (SR141716A) (
Cheer et al., 2007
). Since DA neurons do not express CB1Rs (
Mátyás et al., 2008
), the effects of cocaine are likely presynaptic to these cells. Moreover, as CB1Rs are expressed on GABA axon terminals in the VTA (
Mátyás et al., 2008
,
Riegel and Lupica, 2004
,
Szabo et al., 2002
), we hypothesize that cocaine releases eCBs in the VTA to inhibit GABA release onto DA neurons to disinhibit these cells. To determine whether the eCB-dependent effects of cocaine occur through actions in the VTA, NAc DA transients were measured during intravenous (i.v.) cocaine administration in vivo and the effect of intra-VTA rimonabant tested (
Figure 2
). A one-way ANOVA for all combinations of intra-VTA and i.v. injections revealed a significant treatment effect ($F(4,31) = 29.14$, $p < 0.0001$). Under baseline conditions, NAc DA transients occurred at 0.028 ± 0.007 Hz, and as reported previously, were significantly increased by i.v. cocaine during intra-VTA vehicle infusion (cocaine + vehicle = 0.176 ± 0.01 Hz; $p < 0.0001$, Bonferroni post hoc comparison;
Cheer et al., 2007
). Injection of rimonabant into the VTA prior to i.v. cocaine significantly reduced this increase in DA transients (cocaine + rimonabant = 0.082 ± 0.10 Hz, n = 6 rats; $p < 0.0001$, Bonferroni post hoc comparison;

Figure 2
C). Moreover, intra-VTA rimonabant alone did not alter the frequency of NAc DA transients (0.032 ± 0.003 Hz, p > 0.05, Bonferroni post hoc comparison,
Figure 2C).

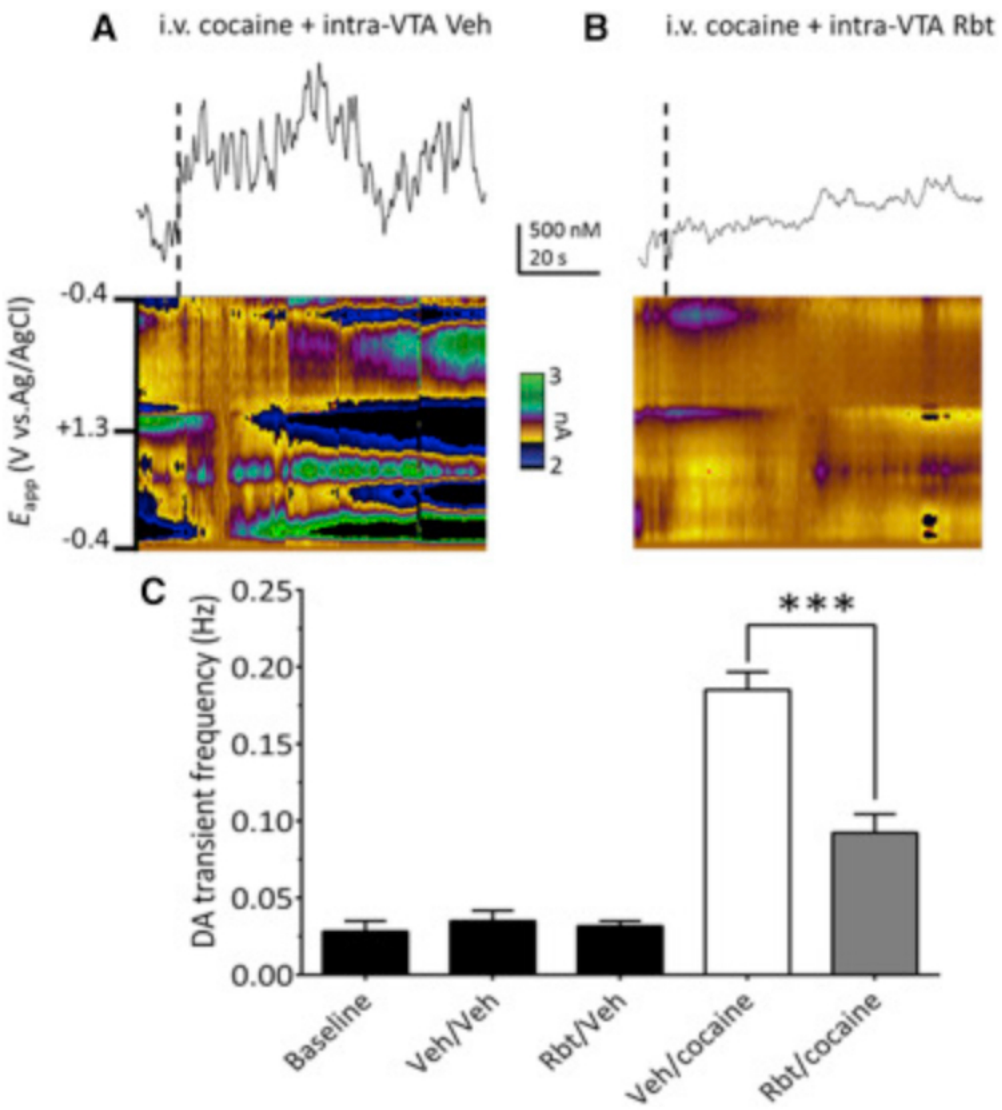


Figure 2. Cocaine-Induced Increase in NAc DA Transients Require CB1R Activation in VTA
(A) Voltammetric current (encoded in color) plotted against the applied carbon-fiber electrode potential (ordinate) and the acquisition time (abscissa). Traces above color plots represent current (normalized to concentration) from the potential where DA is oxidized (~+0.6 V in green), when animals are given an i.v. bolus of cocaine (1 mg/kg), preceded by intra-VTA vehicle infusion.
(B) Systemic effects of cocaine are attenuated following CB1R blockade in VTA (250 ng rimonabant [Rbt] in 500 µl, unilateral and ipsilateral to the recording electrode).
(C) Effects of all treatments on NAc DA transient frequency ($F(4,31) = 29.14$, $p < 0.0001$, one-way ANOVA; $n = 12$ rats). Baseline indicates the frequency of DA transients without intracranial or systemic injections. In other groups the intra-VTA injection is indicated first, followed by the i.v. injection (e.g., intracranial Veh↘intra-VTA Veh). Note that Rbt alone (Rbt/Veh) did not alter DA transient frequency, and Rbt significantly reduced the effect of i.v. cocaine on DA transients (Rbt/cocaine, ***$p < 0.0001$, Bonferroni post hoc test).

2-AG-Mediated Inhibition of GABAB IPSCs by Cocaine

We next determined whether cocaine enhanced eCB release in VTA in vitro and whether this reduced GABAergic input to DA neurons. Cocaine (10 µM) significantly inhibited GABAB IPSCs (67.5% ± 3.0% of control; n = 19; p < 0.0001, paired t test;
Figures 3
A and 3C) and this was significantly reduced by AM251 (2 µM; 87.2% ± 4.8% of control, n = 7; p < 0.01, one-way ANOVA, Bonferroni post hoc analysis;
Figures 3
A and 3C). Furthermore, the effects of cocaine on GABAB IPSCs reversed when its application was stopped (
Figures S1
B and S1C). To determine whether 2-AG was the eCB whose actions were increased by cocaine, we examined the effects of the DGLα inhibitor, THL on the inhibition of GABAB IPSCs produced by cocaine. Cocaine's inhibition of GABAB IPSCs was not significantly reduced by THL (5 µM) when included in the intra-pipette solution (intra-THL: 75.0% ± 2.7% of control; p > 0.05, one-way ANOVA, Bonferroni post hoc analysis, n = 9;
Figures 3
B and 3C). However, extracellular application of THL (10 µM) significantly reduced the inhibitory effect of cocaine on GABAB IPSCs (extra-THL = 85.6% ± 4.7% of control, n = 5; p = 0.009, paired t test; p < 0.05, one-way ANOVA, Bonferroni post hoc analysis;
Figures 3
B and 3C). This suggests that cocaine releases 2-AG from sites that are more widely distributed than those involved in tonic inhibition of GABAB IPSCs under control conditions (Figure 1C).

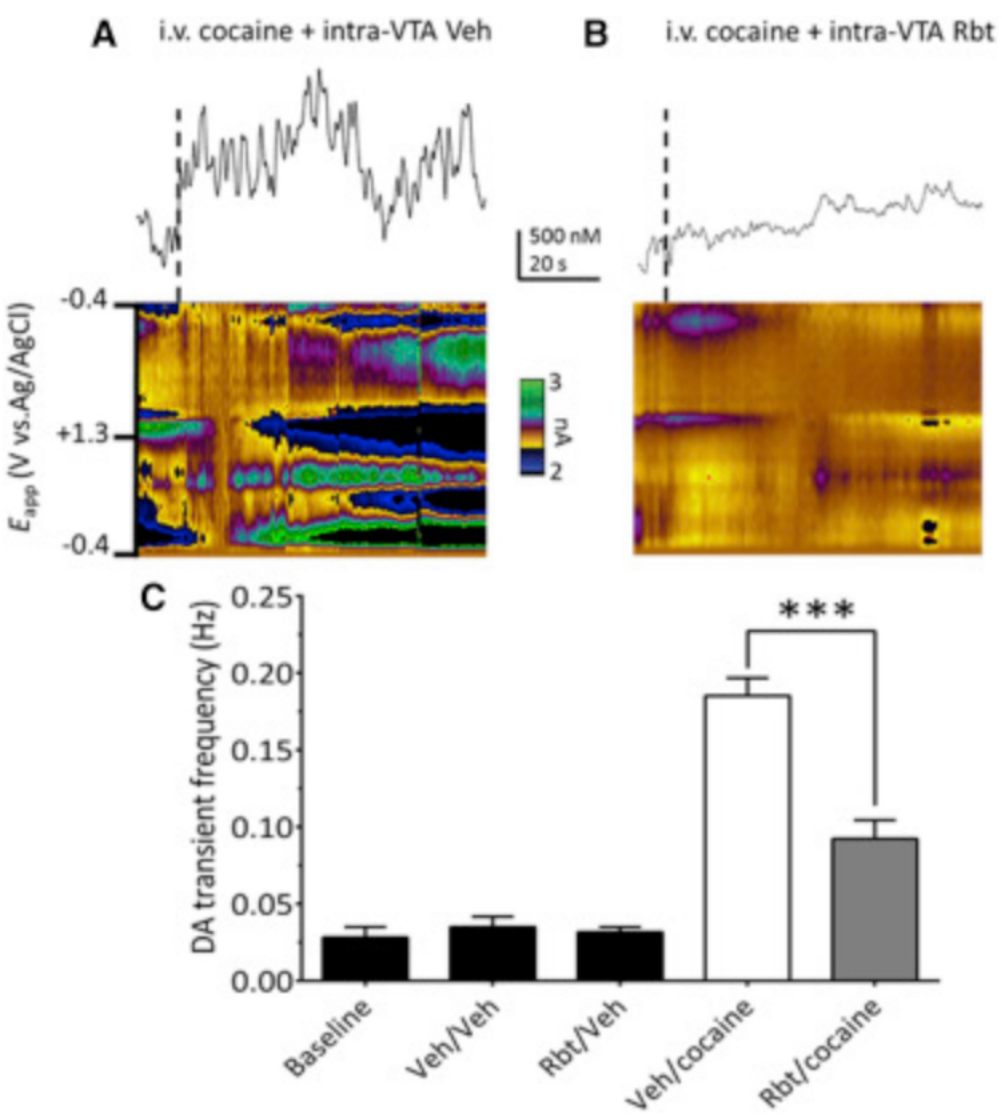


Figure 3. Cocaine-Mobilized 2-AG Inhibits GABA Release and Increases DA Release in NAc via Actions in VTA

(A) Acute cocaine (10 μM) perfusion inhibited GABAB IPSCs and this was partially blocked by AM251 (2 μM). Scale bar, 100 ms, 10 pA.

(B) Cocaine inhibition of GABAB IPSCs was reduced by inhibition of DGL with THL, applied either intracellularly via the pipette (intra-THL) or extracellularly, by addition to the aCSF (extra-THL). Scale bar, 100 ms, 10 pA.

(C) Summary of the experiments shows significant reduction of the cocaine inhibition of GABAB IPSCs by AM251 and THL ($n = 5$; $F(3,36) = 6.35$, $p = 0.0014$, one-way ANOVA; $*p < 0.05$ and $**p < 0.01$, Bonferroni post hoc analysis).

(D) Cocaine did not affect postsynaptic GABABcurrents evoked by laser (indicated by vertical bar on sweeps inset) uncaging of O-CNB-Caged-GABA (100–200 μM in bath; $n = 7$, $p > 0.05$, paired t test. Scale bar, 100 ms, 10 pA.

(E) In vivo NAc FSCV current (encoded in color) is plotted against the applied potential (ordinate) and the acquisition time (abscissa). Traces above color plots are extracted currents (normalized to concentration) from the potential where DA is oxidized (~+0.6 V shown in green), when animals are given intravenous cocaine (1 mg/kg), preceded by an i.c.v. vehicle injection.

(F) Effects of cocaine are reduced in vivo by inhibition of 2-AG synthesis by THL in the VTA (750 ng THL in 500 μl, unilateral and ipsilateral to the recording electrode).

(G) Intra-VTA THL reduces DA transient frequency elicited by i.v. cocaine ($F(4,10) = 9.395$, $p = 0.002$, one-way ANOVA; $n = 3$ rats, $**p < 0.001$, Bonferroni post hoc analysis). Baseline indicates DA transient frequency without intracranial or systemic injections. In other groups, the intra-VTA injection is indicated first, followed by the i.v. injection condition (e.g., intracranial Veh↘intra-VTA Veh). Note that THL alone (THL/Veh) did not alter DA transient frequency, and THL significantly reduced the effect of i.v. cocaine on DA transients (THL/cocaine, $p < 0.001$, Bonferroni post hoc test).

See also

Figure S1

.

The main site eCBs act in the CNS is at CB1Rs on axon terminals to suppress neurotransmitter release (

Kreitzer and Regehr, 2001

,

Ohno-Shosaku et al., 2001

,

Wilson and Nicoll, 2001

). To determine whether eCB-dependent effects of cocaine on GABAB IPSCs were presynaptic we measured GABA-mediated postsynaptic currents elicited by laser (355 nm) photolysis of *O*-(CNB-Caged) GABA (100–200 μM). Light-evoked GABABcurrents were not altered by cocaine (control: 23.79 ± 4.85 pA; cocaine: 22.38 ± 4.74 pA; $n = 7$; $p > 0.05$, paired t test;

Figure 3

D), suggesting that inhibition of electrically evoked GABAB IPSCs by cocaine occurred presynaptically.

We next determined whether 2-AG in the VTA mediates the increase in NAc DA transients during cocaine application in vivo using THL injection in VTA. A one-way ANOVA for all combinations of intra-VTA and i.v. injections revealed a significant treatment effect ($F(4,10) = 9.395$, $p = 0.002$). Under baseline conditions, DA transients occurred at 0.044 ± 0.003 Hz and this was significantly increased by i.v. injection of cocaine and intra-VTA vehicle infusion (cocaine + vehicle = 0.094 ± 0.007 Hz; $p < 0.001$, Bonferroni post hoc comparison,

Figure 3
G). Intra-VTA THL significantly reduced the increase in DA transients by cocaine (cocaine + THL = 0.05 ± 0.011 Hz, n = 3 rats; $p < 0.001$, Bonferroni post hoc comparison;
Figure 3
G). Moreover, intra-VTA THL alone did not alter the frequency of DA transients, compared to pre-cocaine baseline (0.045 ± 0.006 Hz, $p > 0.05$, Bonferroni post hoc comparison,
Figure 3
G). Consistent with the in vitro experiments, these data suggest that cocaine enhances 2-AG levels in the VTA to disinhibit DA neurons, contributing to increased DA release in the NAc.

Cocaine-Induced 2-AG Signaling Is Independent of 5-HT and DA

Our study shows that AM251 and THL do not completely prevent inhibition of GABA release produced by cocaine (
Figure 3
), and another study shows that cocaine inhibits GABA release onto DA neurons in VTA by inhibiting serotonin (5-HT) reuptake and activation of 5-HT1B/D receptors (
Cameron and Williams, 1994
). Therefore, we determined whether the effects of cocaine on 2-AG involved 5-HT. The 5-HT1 receptor agonistsumatriptan (300 nM) decreased GABAB IPSCs (71.42% ± 4.50% of control, n = 8) and largely occluded the inhibition produced by cocaine (cocaine alone = 67.53% ± 3.01% of control, n = 19; sumatriptan + cocaine = 91.51% ± 3.52% of control, n = 8; $p < 0.001$, one-way ANOVA, Bonferroni post hoc analysis;
Figures 4
A, 4D, and 4G). The 5-HT1B/Dantagonist, GR55562 (1 or 10 μM) had no effect alone on GABAB IPSCs but decreased the inhibition of this response by cocaine (cocaine alone = 67.53% ± 3.01% of control, n = 19; GR55562 + cocaine = 83.94% ± 3.91% of control, n = 10; $p < 0.05$, one-way ANOVA, Bonferroni post hoc analysis;
Figures 4
B and 4G). Additionally, the 5-HT uptake inhibitor citalopram (10 μM) and the 5-HT releaser fenfluramine (10 μM) both depressed GABAB IPSCs (citalopram: 77.77% ± 5.6% of control, n = 7; fenfluramine: 67.06% ± 3.42% of control, n = 11;
Figure 4D).

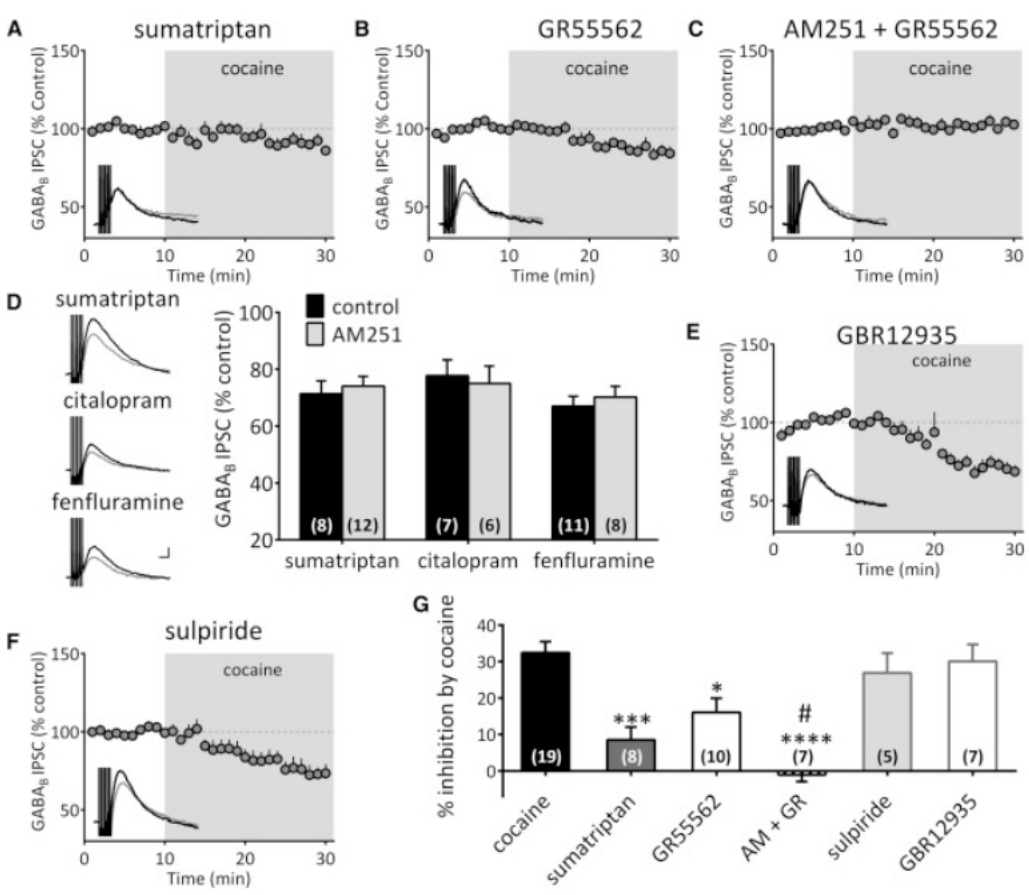


Figure 4. Inhibition of GABAB IPSCs by Cocaine Is Independently Mediated by 5HT1B and CB1 Receptors
(A) Inhibition of GABAB IPSC by cocaine was largely occluded by the 5HT1B receptor agonist sumatriptan (300 nM).
(B) The selective 5HT1B receptor antagonistGR55562 (1 or 10 μM) suppressed the inhibition by cocaine.
(C) The inhibition by cocaine was completely abolished when CB1 and 5HT1B receptors were blocked simultaneously.
(D) Serotonin-induced depression of GABAB IPSC is independent of CB1Rs. Application of the 5HT1Bagonist sumatriptan, the selective serotonin reuptake inhibitor citalopram (10 μM), or the serotonin releaser fenfluramine (10 μM) all depressed GABAB IPSCs to a similar level. However, this is unaffected by the CB1R antagonist AM251 (4 μM; $F(2,46) = 0.25$, $p = 0.77$, two-way ANOVA). Scale bar, 100 ms, 10 pA.
(E) Pre-treatment with the selective DA transport inhibitor, GBR12935 (1 μM), does not occlude the inhibition of GABAB IPSCs by cocaine.
(F) The DA-D2 receptor antagonist sulpiride (5 μM) does not prevent the inhibition by cocaine.
(G) Summary the effect of cocaine on GABAB IPSCs with different drug treatments ($F(5,51) = 12.83$, $p < 0.0001$, one-way ANOVA; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ versus cocaine; $\#p < 0.05$ versus GR55562; Bonferroni post hoc analysis). The complete elimination of the cocaine-induced inhibition of GABAB IPSCs by CB1R and 5-HT1Bantagonism indicates that these effects are independent.
To determine whether the inhibition of GABABIPSCs by the 5-HT system involves eCBs, we tested the effects of citalopram and fenfluramine

during AM251 application (
Figure 4
D). We found that inhibition of GABAB IPSCs produced by these drugs was unaffected by CB1R antagonism (citalopram + AM251 = 75.03% ± 6.10% of control, n = 6; fenfluramine + AM251 = 70.26% ± 3.74% of control, n = 8; $p > 0.05$, unpaired t test;
Figure 4
D). Similarly, the effect of the 5-HT1 agonist sumatriptan was not altered by AM251 (sumatriptan + AM251 = 74.07% ± 3.41% of control, n = 12;
Figure 4
D). Therefore, we conclude that whereas cocaine enhances extracellular 5-HT levels to activate 5-HT1 receptors and inhibit GABA release onto DA neurons, this is independent of the inhibition produced by cocaine via 2-AG. Therefore, the inhibition of GABA release by cocaine in the VTA occurs via 2 independent mechanisms. Consistent with this, co-application of the 5-HT1B/D antagonist GR55562 and AM251 completely blocked the inhibition of GABAB IPSCs produced by cocaine (AM251 + GR55562 + cocaine = 101.09% ± 1.88% of control, n = 8; $p < 0.0001$ versus cocaine, one-way ANOVA;
Figures 4
C and 4G). Therefore, all subsequent experiments examining cocaine mobilization of 2-AG, were performed during blockade of 5-HT1B/1D receptors with GR55562 (1 μM).
Previous studies show that DA-D2 receptors stimulate 2-AG release (
Melis et al., 2004
,
Pan et al., 2008
). Therefore, we examined the involvement of D2 receptors and DA in 2-AG-mediated cocaine effects. In contrast to cocaine, the selective DAT inhibitor GBR12935 (1 μM) increased GABAB IPSCs and surprisingly did not occlude their inhibition by cocaine (GBR12935 + cocaine = 69.93% ± 4.67% of control, n = 7;
Figures 4
E and 4G). Similarly, a DA-D2 antagonist, sulpiride (5 μM), did not change the inhibition of GABAB IPSCs produced by cocaine (73.09% ± 5.45% of control, n = 5;
Figures 4
F and 4G). Therefore, the cocaine-induced inhibition of GABAB IPSCs did not result from increased extracellular DA levels or activation of DA-D2 receptors.

Gq/11-Coupled Receptor Involvement in the Cocaine-Induced Release of 2-AG

Several studies show that Gαq/11-coupled receptors promote the production of 2-AG in the brain (
Kano et al., 2009
,
Kim et al., 2002
,
Kortleven et al., 2011
,
Maejima et al., 2001
). Therefore, we examined their involvement in cocaine-induced 2-AG mobilization. Antagonists for Gq/11-coupled mGluR5, DA-D1, and neurotensin receptors did not alter the cocaine-induced inhibition of GABAB IPSCs in VTA DA neurons (
Figure S2
). However, antagonism of Gq/11-coupled α1-adrenergic receptors (α1R) with 2-[[β-(-4-hydroxyphenyl)ethyl]aminomethyl]-1-tetralone) (HEAT, 1 μM) or the selective, non-competitive, mGluR1 receptor antagonist, (3,4-dihydro-2H-pyrano[2,3-b]quinolin-7-yl)-(*cis*-4-methoxycyclohexyl)-methanone (JNJ16259685 [JNJ]; 500 nM) completely blocked the 2-AG-dependent inhibition of GABAB IPSCs promoted by cocaine (cocaine = 83.94% ± 3.91% of control, n = 10; cocaine + HEAT = 99.68% ± 4.73% of control, n = 7; cocaine + JNJ = 99.39% ± 4.49% of control, n = 8; $p < 0.01$, one-way ANOVA, Bonferroni post hoc analysis;
Figure 5
A). This indicates that activation of both mGluR1 and α1Rs during cocaine application is required for the 2-AG-dependent inhibition of GABA release. We next examined the involvement of these receptors using selective agonists.

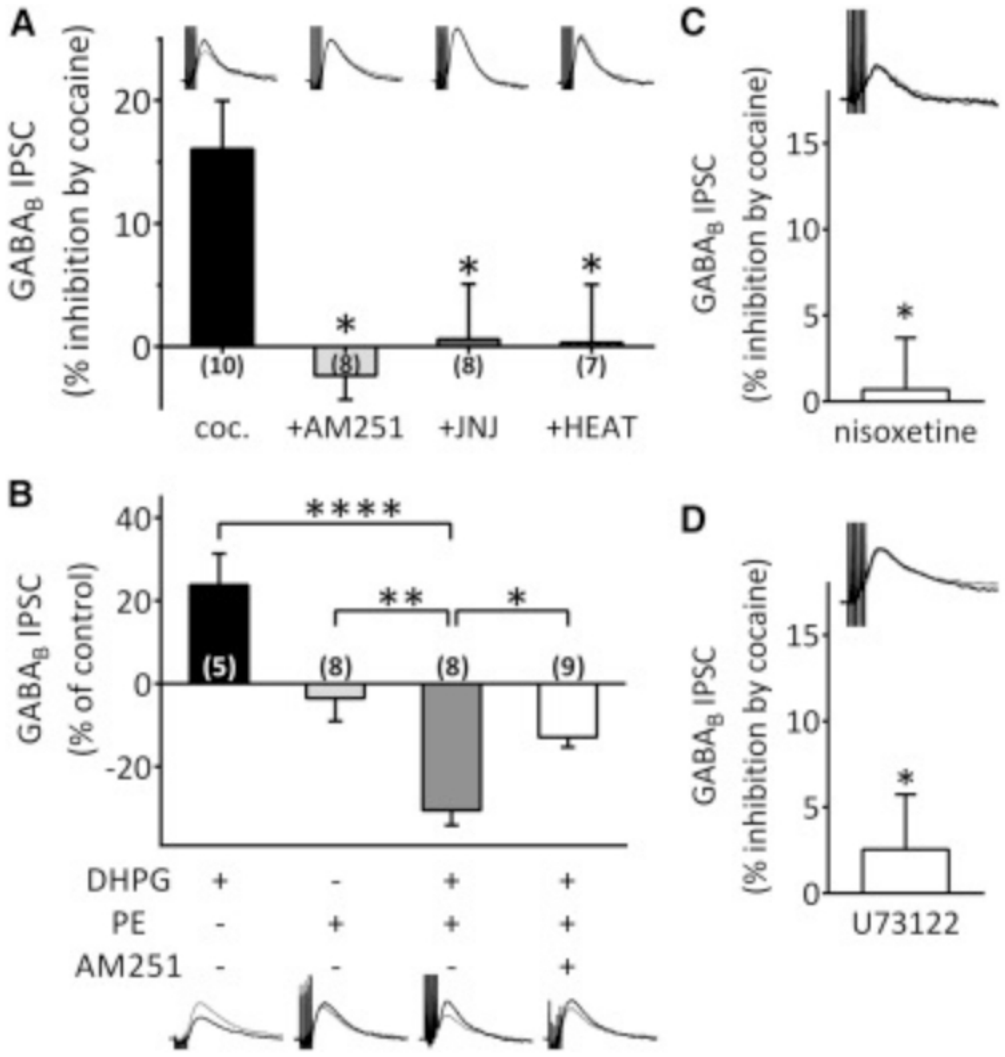


Figure 5. Mobilization of 2-AG by Cocaine Requires Synergistic Activation of mGluR1 and α1 Adrenergic Receptors and Activation of Phospholipase C

All experiments were performed in the presence of GR55562 to block 5-HT1B receptors.

(A) Inhibition of GABAB IPSCs by cocaine was blocked by AM251 (2 μM), the mGluR1 antagonist JNJ16259685 (JNJ, 500 nM) and the α1R antagonist HEAT (1 μM) ($F(3,29) = 5.173$, $p = 0.0055$, one-way ANOVA; $*p < 0.05$, Bonferroni post hoc analysis).

(B) Co-application of the mGluR1 agonist DHPG (1 μM) and the α1 agonist phenylephrine (PE, 100 μM) inhibited GABAB IPSCs, whereas alone these agonists did not exhibit such effects. Pre-incubation of slices with AM251 significantly blocked the synergistic effect of DHPG and PE ($F(3,26) = 20.19$, $p < 0.0001$, one-way ANOVA; $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$, Bonferroni post hoc analysis). Scale bar, 100 ms, 10 pA. Scale bar applies to (A).

(C) The NET inhibitor nisoxetine (10 μM) occluded the inhibition of GABAB IPSCs by cocaine ($*p < 0.05$, unpaired t test). Scale bar, 100 ms, 10 pA.

(D) An inhibitor of PLC, U73122 (5 μM), significantly blocked the effect of cocaine ($*p < 0.05$ versus cocaine, unpaired t test). Scale bar, 100 ms, 10 pA.

See also

Figures S2

and

S3

.

The mGluR1 agonist, DHPG (1 μM), alone, significantly increased the amplitude of GABABIPSCs (123.80% ± 7.58% of control, n = 5; $p < 0.05$, paired t test;

Figure 5

B), whereas the α1R agonist, phenylephrine (PE, 100 μM) did not affect these currents (96.52% ± 5.60% of control, n = 8; $p = 0.52$, paired t test;

Figure 5

B). In contrast, co-application of DHPG and PE strongly inhibited GABAB IPSCs (69.53% ± 3.66% of control, n = 8; $p < 0.001$, paired t test;

Figure 6

B) and this was significantly reduced by AM251 pretreatment (87.09% ± 2.36% of control, n = 9; $p < 0.05$ versus HEAT + PE, unpaired t test;

Figure 5

B). This suggests that co-activation of Gq/11-coupled mGluR1 and α1Rs is necessary for cocaine to promote 2-AG mobilization and inhibit GABA release onto VTA DA neurons.

As cocaine is a potent inhibitor of the norepinephrine transporter (NET), we next examined whether the α1R-dependent effect of cocaine on GABAB IPSCs occurred via this mechanism. The selective NET inhibitor, nisoxetine(10 μM), completely blocked the inhibition of IPSCs produced by cocaine (99.31% ± 3.00% of control, n = 7; $p < 0.05$ versus cocaine, unpaired t test,

Figure 5

C), suggesting that cocaine promotes activation of α1Rs and the mobilization of 2-AG through the inhibition of NET.

Contribution of PLC-β and Intracellular Ca2+ to the Cocaine Mobilization of 2-AG

The lipolytic enzyme, PLC-β, promotes conversion of phosphoinositides to DAG, which can then be converted to 2-AG via DGLα (

Alger and Kim, 2011
,
Hashimotodani et al., 2005
,
Kano et al., 2009
). As PLCβ is activated by a rise in internal Ca2+, or activation of Gq/11-coupled receptors, we determined whether PLC-β inhibition alters the 2-AG inhibition of GABA release by cocaine. The cocaine inhibition of GABAB IPSCs was absent in DA neurons incubated in the PLC inhibitor, U73122(5 μM; cocaine + U73122 = 97.46% ± 3.20% of control, n = 7; $p < 0.05$, unpaired t test,
Figure 5
D), suggesting that cocaine-induced 2-AG mobilization requires PLC.
To assess the role of Ca2+ in the release of 2-AG by cocaine, in vitro calcium imaging experiments were performed in VTA DA neurons expressing the genetically-encoded calcium indicator, GCaMP6f, in TH-Cre rats. Robust expression of GCaMP6f was confirmed (
Figure 6
A), and DA neurons exhibiting stable Ca2+-associated fluorescence and spontaneous Ca2+ oscillations were observed (
Figures 6
A and 6B;
Movie S1
). The mean frequency of spontaneous calcium oscillations in a sub-group of neurons (29.1%, 168 of 578 neurons) (
Figure 6
B;
Movie S1
) under resting conditions was 0.042 ± 0.002 Hz (range 0.0026–0.167 Hz,
Figures 6
H and 6I). With bath application of cocaine (10 μM), the Ca2+ signal in 21.2% (32 of 151) of the DA neurons was unchanged, whereas it was reduced by cocaine in 59.6% (90 of 151) of DA neurons (
Figures 6
C, 6D, and 6J;
Movie S1
). Among this latter population, 47.8% (43 of 90) exhibited slow calcium oscillationsduring baseline whose frequency was reduced by cocaine (control = 0.041 ± 0.004 Hz; cocaine = 0.012 ± 0.003 Hz; $p < 0.0001$, paired t test;
Figures 6
C–6E). The remaining neurons (19.2%, 29 of 151) exhibited no Ca2+ oscillations during baseline but demonstrated oscillations during cocaine application (0.023 ± 0.004 Hz; $p < 0.01$ versus spontaneous oscillation, Kolmogorov-Smirnov [K-S] test;
Figures 6
C–6E and 6J). These cocaine-induced slow Ca2+ oscillations (SO) exhibited lower frequencies and broader half-widths than oscillations observed during baseline (spontaneous baseline oscillation half-width = 11.21 ± 2.48 s; cocaine-induced oscillation half-width = 24.17 ± 3.19 s; $p < 0.0001$, K-S test;
Figure 6
F), suggesting that cocaine SOs reflect distinct Ca2+ sources and/or release mechanisms. The cocaine SOs were also more highly synchronized among VTA DA neurons than the spontaneous Ca2+ oscillations under baseline conditions, as indicated by cross correlation analysis (
Figure 6G).

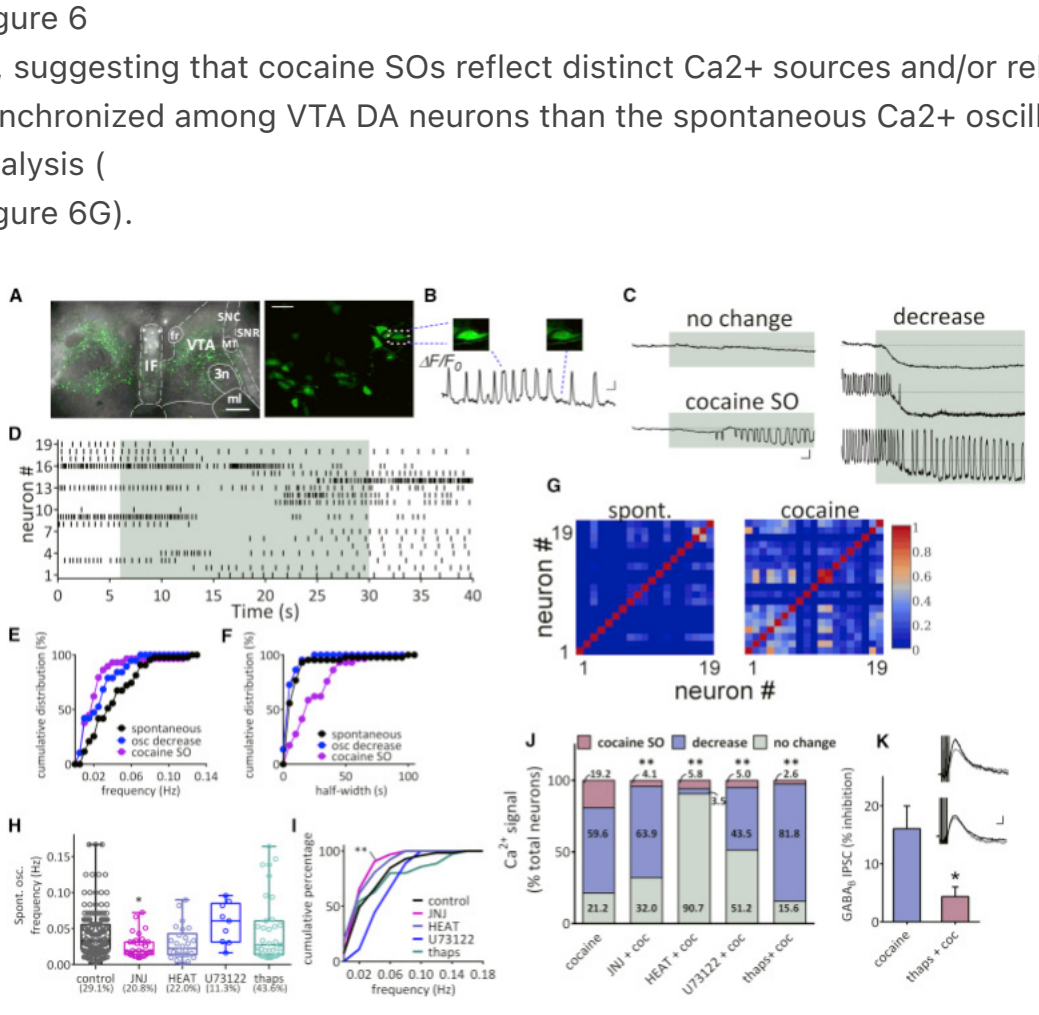


Figure 6. Cocaine-Induced Calcium Changes in VTA DA Neurons
(A) Superimposed fluorescent and transmitted light images showing the expression of GCaMP6f in VTA DA neurons 2–3 weeks after

injection of the AAV- GCaMP6f construct. Scale bar, 500 μm.
(B) Spontaneous slow calcium oscillations in VTA DA neurons. Left: confocal image of the GCaMP6f signal (200×). Scale bar, 50 μm. Right: example calcium oscillations (ΔF/F0) in the same cell indicated in the dashed square at left. Scale bar, 30 s, 20%.
(C) Sample traces of calcium signals (ΔF/F0) in response to cocaine application (10 μM, indicated in gray). Three types of changes were observed: no change, a decrease in spontaneous oscillations, and the initiation of slow oscillations (SO).
(D) Raster plot of oscillation events in 19 neurons imaged as in (B). Each vertical bar denotes the peak of an oscillation event. Each row exhibits the time course of oscillation change in single neurons.
(E) Cumulative frequencies of spontaneous, cocaine-decreased, and cocaine-initiated slow oscillations (cocaine SO) in calcium signal. The curve shifts left to slower frequencies in neurons whose calcium oscillations were decreased by cocaine (osc decrease versus spontaneous, $p = 0.17$, K-S test). The frequency of cocaine-initiated SO was significantly lower than that of spontaneous oscillations (cocaine-initiated versus spontaneous, $p < 0.01$, K-S test). Spontaneous oscillation, n = 43 neurons; oscillation decrease, n = 19 neurons; cocaine SO, n = 29 neurons recorded in eight brain slices from six rats.
(F) Cumulative distribution of half-widths of spontaneous, cocaine-decreased, and cocaine-initiated SO. Cocaine-initiated SO exhibit longer half-widths than spontaneous oscillations. (cocaine SO versus spontaneous, $p < 0.0001$, K-S test). Spontaneous oscillation, n = 43 neurons; oscillation decrease, n = 19 neurons; cocaine SO, n = 29 neurons recorded in eight brain slices from six rats.
(G) Increased correlation of calcium responses among neurons (same 19 neurons as D) during cocaine perfusion. Cross correlations among all neuron pairs were calculated, and the peak correlation coefficient (r = 0 to 1) is color-coded to form the heat map. The data indicate that VTA DA neuron calcium oscillations are more highly synchronized by cocaine.
(H) Cocaine-induced calcium oscillations depend on mGluR1, α1-adrenergic receptors, and internal calcium stores. The frequency of spontaneous calcium oscillations is reduced by JNJ16259685 (500 nM), but is not altered by HEAT (1 μM), U73122, or thapsigargin (thaps, 2 μM), respectively ($F(4,265) = 3.806$, $p = 0.005$, one-way ANOVA; control versus JNJ: $p < 0.05$, Bonferroni post hoc analysis). Each point represents the frequency of spontaneous Ca2+ oscillation from one neuron; the box and vertical lines indicate quartiles, minimal, and maximal frequencies. The percentage of neurons exhibiting spontaneous oscillations is shown for each treatment group.
(I) Cumulative distributions of the frequencies of spontaneous oscillations are only affected by JNJ ($p = 0.0037$, Kolmogorov–Smirnov test).
(J) Summary of the percentage of DA neurons responding to cocaine under control, JNJ16259685, HEAT, U73122, and thapsigargin treatments. Numbers denote the percentage of neurons demonstrating indicated changes upon cocaine treatment. All treatments decreased the cocaine-induced Ca2+ oscillation.
(K) Pre-treatment of slices with thapsigargin (2 μM) significantly attenuates the inhibition of GABABIPSCs by cocaine (n = 7; $*p < 0.05$, unpaired t test). Inset at right shows averaged electrically evoked GABAB IPSC traces (n = 5) under control conditions (top) and after treatment with thapsigargin. Control sweeps are shown in black; those obtained during cocaine application are shown in gray. Scale bar, 100 ms, 10 pA.
See also
Movie S1
.
In electrophysiological experiments, the cocaine-induced depression of GABA release was mediated by 2-AG and abolished by antagonism of mGluR1 or α1Rs (
Figure 5
A). Therefore, we examined effects of these antagonists on cocaine-induced changes in intracellular Ca2+. The mGluR1 antagonist, JNJ16259685 (500 nM), prevented the cocaine-induced increase in the Ca2+ SO, as only 4.1% (4 of 97 cells) of DA neurons showed this response (
Figure 6
J, $**p < 0.0001$, chi-square test). Moreover, the proportion of DA neurons showing no change in intracellular Ca2+ with cocaine increased to 32.0% (31 of 97 neurons,
Figure 6
J), and the proportion of VTA DA neurons exhibiting a decrease in Ca2+ signal upon cocaine application was not altered by JNJ162959685 (63.9%, 62 of 97 neurons,
Figure 6
J). The α1R antagonist, HEAT (1 μM), also prevented the increased the cocaine-induced Ca2+ SO, as only 5.8% (5 of 86 neurons) showed this response (
Figure 6
J, $p < 0.0001$, chi-square test). However, unlike the mGluR1 antagonist, HEAT also reduced the proportion of neurons exhibiting a decrease in Ca2+ oscillations (3.5%, 3 of 86,
Figure 6
J). The PLCβinhibitor, U73122, also prevented the cocaine-induced SO, as only 5.0% (4 of 80 cells,
Figure 6
J, $p < 0.0001$, chi-square test) of the DA neurons showed this effect.
The number of neurons showing an increase in Ca2+SO with cocaine was also reduced when intracellular stores were depleted with the sarco/endoplasmic reticulum Ca2+ ATPase inhibitor, thapsigargin (2 μM; oscillation increase in 2.6%, 2 of 77 neurons,
Figure 6
J, $p < 0.0001$, chi-square test). Additionally, the proportion of neurons showing inhibition of the Ca2+ signal by cocaine was increased

following thapsigargin (81.8%, 63 of 77 neurons;
Figure 6
J). The frequency of baseline Ca2+oscillations was significantly reduced by JNJ162959685 (p = 0.0037 versus cocaine, K-S Test,
Figures 6
H and 6I), but unchanged by HEAT, U73122, or thapsigargin (
Figures 6
H and 6I). However, the percentage of neurons showing these oscillations in the absence of cocaine was lower in U73122-treated slices and higher in thapsigargin-incubated slices (control: 29.1%; HEAT: 22.0%; JNJ: 20.8%; U73122: 11.3%; thapsigargin: 43.6%;
Figure 6
H).

Depletion of Internal Ca2+ Stores Blocks the 2-AG-Dependent Effects of Cocaine

To determine whether elimination of the cocaine-induced Ca2+ SOs also blocks the cocaine-mediated release of 2-AG, we measured GABAB IPSCs after treatment with thapsigargin (2 µM;
Figure 6
K). We found that thapsigargin prevented the cocaine inhibition of GABAB IPSCs in DA neurons (95.63% ± 1.67% of control, n = 7; p < 0.05 versus cocaine alone, unpaired t test;
Figure 6
K), confirming that internal Ca2+ is necessary for cocaine-induced 2-AG mobilization.

Discussion

Here, we find that cocaine mobilizes 2-AG in the VTA to inhibit GABAergic inputs to GABAB receptors on DA neurons via presynaptic CB1R activation. We also show that cocaine's ability to increase the frequency of DA transients in the NAc is dependent on 2-AG synthesis and CB1R signaling in the VTA, collectively implicating an eCB-dependent disinhibition of VTA DA neurons. Our results thereby provide a mechanism to explain the disruption of cocaine-motivated behavior that is observed during antagonism of the eCB system in animal models of addiction and relapse to drug seeking (
De Vries et al., 2001
,
Li et al., 2009
,
Soria et al., 2005
,
Xi et al., 2006
,
Xi et al., 2008
). Based on these findings, we hypothesize that cocaine-induced 2-AG mobilization in the VTA facilitates DA release in the NAc to support cocaine reinforcement.

The cocaine-induced inhibition of GABA release is disrupted by several manipulations that target the eCB system in the VTA, including (1) blockade of CB1Rs, (2) inhibition of PLCβ, and (3) inhibition of DGLα. We also find that 2-AG mobilization by cocaine requires activation of both mGluR1 and α1Rs, since antagonism of either receptor prevents inhibition of GABAB IPSCs in DA neurons. As the inhibition of GABA release by cocaine was blocked by a NET inhibitor, our data further suggest that cocaine increases extracellular NE and activates α1Rs through this mechanism. Prior studies show that cocaine increases NE via inhibition of NET (
Reith et al., 1997
), and NE neurons project to the VTA to form connections with DA neurons (
Jones et al., 1977
,
Mejías-Aponte et al., 2009
). Moreover, genetic elimination of α1Rs is associated with lower baseline levels of DA and prevents psychostimulant-mediated increases in NAc DA (
Auclair et al., 2002
). A more recent study shows that cocaine increases extracellular NE in VTA to activate α1Rs, contributing to excitation of DA neurons and DA release in the NAc (
Goertz et al., 2014
). This, together with our data, suggests that modulation of NE function in the VTA likely plays a critical role in the behavioral effects of cocaine.

We also find that mGluR1s are critical for the 2-AG-dependent effects of cocaine in the VTA. However, electrically-evoked EPSCs in VTA DA neurons are unaffected by cocaine under our recording conditions (
Figure S3
), suggesting that synaptic glutamate release is not involved. Despite this, prior work shows that cocaine increases glutamateconcentrations measured via microdialysis (
Kalivas and Duffy, 1995

), and mGluR1s are activated by glutamate in DA neurons using electrical stimulation similar to ours (
Fiorillo and Williams, 1998
). Therefore, we suggest that sufficient extracellular glutamate is present under baseline conditions to activate mGluR1s and this cooperatively interacts with α1Rs to stimulate 2-AG production.

Imaging of Ca2+ dynamics in TH-Cre rats with GCaMP6f reveals that cocaine initiates Ca2+ SOs in ~20% of VTA DA neurons, whereas baseline spontaneous Ca2+ oscillations are suppressed in most cells. Antagonism of either mGluR1s or α1Rs with JNJ16259685 or HEAT, respectively, largely eliminates the cocaine SOs; an effect also seen with blockade of PLCβ by U73122 or depletion of intracellular Ca2+ by thapsigargin. Corresponding blockade of both cocaine-induced SOs and inhibition of GABAB IPSCs by each of these manipulations supports the idea that the 2-AG-dependent actions of cocaine are related to an increase in Ca2+ activity and dependent on cooperative mGluR1 and α1AR signaling.

The activity of PLCβ is enhanced in neurons by intracellular Ca2+ and this is synergistically increased by co-activation of Gq/11-coupled receptors to greatly augment 2-AG mobilization (
Alger and Kim, 2011
,
Kano et al., 2009
). Here, we show that cocaine causes activation of Gq/11-coupled α1Rs in DA neurons through an increase in extracellular NE via inhibition of NET. The requirement for co-activation of mGluR1 and α1Rs suggests that there is an interaction at the Gq/11-coupled receptor level that increases the activity of PLCβ to synergistically augment 2-AG release. This cooperativity between mGluR1 and α1Rs in the inhibition of GABA release onto VTA DA neurons may serve as a coincidence detector, such that when sufficient NE is present, together with glutamate activation of mGluR1s, 2-AG is mobilized to reduce GABAergic input, thereby enhancing DA neuron activity via disinhibition. Consequently, this will amplify forebrain DA release, as shown here, and potentially enhance the reinforcing properties of cocaine.

Our data also suggest that the cocaine-induced 2-AG mobilization occurs in the ~20% of VTA DA neurons demonstrating cocaine-induced Ca2+oscillations. This, together with our observation that extracellular application of THL is more effective at blocking the cocaine-induced inhibition of GABA release than intracellular application, supports the idea that 2-AG is released from this subset of neurons to more widely affect synaptic inputs. Moreover, since GCaMP6f was expressed only in DA neurons in our study, the possibility that cocaine releases 2-AG from non-DA neurons was not evaluated and therefore remains a possibility.

The rewarding and abuse-related properties of cocaine are thought to primarily result from inhibition of DA uptake in forebrain areas such as the NAc (
Phillips et al., 2003
,
Ritz et al., 1987
,
Sulzer, 2011
,
Thomsen et al., 2009
). However, cocaine also acts in the VTA to increase DA neuron firing rates and DA release events in the NAc (
Sombers et al., 2009
), and these events are decreased by systemic CB1R antagonists (
Cheer et al., 2007
). DA transients result from burst firing that occurs upon presentation of natural reward, cues that predict their availability or during administration of abused drugs (
Aragona et al., 2008
,
Cheer et al., 2007
,
Oleson et al., 2012
,
Phillips et al., 2003
,
Roitman et al., 2004
,
Sombers et al., 2009
) and function to reinforce behavior (
Steinberg et al., 2014
). Here, we demonstrate that the eCB influence on DA transients is likely mediated by 2-AG acting at CB1Rs in the VTA, since intra-VTA administration of a CB1R antagonist, or an inhibitor of DGLα blocked this effect of cocaine. Although inhibition of VTA DA neurons is the most frequent response to cocaine in anesthetized rodents, an increase in the bursting activity of a proportion of these cells is consistently reported (
Einhorn et al., 1988
,

Mejias-Aponte et al., 2015
). Furthermore, a much larger proportion of VTA DA neurons is excited by cocaine in awake-freely moving rats, compared to anaesthetized animals (
Koulchitsky et al., 2012
). Our lab has shown that that conditions promoting burst firing of DA neurons causes activity- and extracellular-Ca2+-dependent eCB release that presynaptically inhibits GABA release onto these cells (
Riegel and Lupica, 2004
). In accord with the present observations that cocaine mobilizes 2-AG via Gq11-dependent mechanisms to enhance the inhibition of GABA inputs to these cells, we suggest that the eCB system in the VTA is an important component of a positive feedback mechanism that is engaged during exposure to cocaine and contributes to its rewarding properties. In this scheme (
Figure 7
), we propose that blockade of NET by cocaine promotes activation of Gq/11-coupled α1Rs and this, together with mGluR1 activation, initiates 2-AG release, which then suppresses GABA release to disinhibit VTA DA neurons. This disinhibition then promotes burst firing of DA neurons, mobilizing additional 2-AG through an activity-dependent mechanism. This then supports further activation of these cells, via disinhibition, to augment DA neuron activity and DA release in downstream projections. In this way, the eCB system promotes strong activation of DA neurons to contribute to the rewarding actions of cocaine. Therefore, the mechanisms that we have identified in the present study may be critical in the development of pharmacotherapies for psychostimulant addiction that target the eCB system.

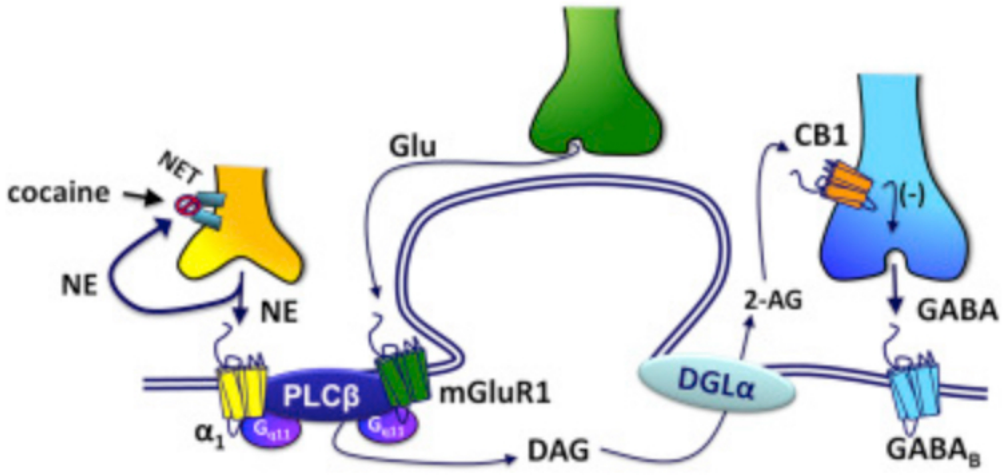


Figure 7. Putative Mechanism of Cocaine Mobilization of 2-AG and Inhibition of GABA Neurotransmission in the VTA
Glutamate spillover under baseline conditions activates mGluR1s, and α1-adrenergic receptors located on VTA DA neurons are activated by NE whose clearance is prevented by cocaine blockade of the NET. This co-activation of Gq11-coupled mGluR1 and α1Rs cooperatively stimulates PLC-β leading to DAG formation. DAG is then converted to 2-AG, via DGL-α, then crosses the extracellular space through an unknown mechanism to activate CB1Rs located on GABA axon terminals. Activation of these presynaptic CB1Rs inhibits GABA release onto postsynaptic GABAB receptors, leading to 2-AG-mediated disinhibition of DA neurons. This increases DA transients in the NAc to enhance the rewarding properties of cocaine.

Experimental Procedures

Animal protocols were conducted under NIH guidelines and were approved by the NIDA Intramural Research Program Animal Care and Use Committee.

Slice Preparation

Male Sprague-Dawley rats, 15 to 21 days old (Charles Rivers) were decapitated, their brains rapidly removed and transferred to an aerated (95% O2/5% CO2), ice-cold solution (in mM: sucrose 220, KCl 2.5, MgCl2 7, CaCl2 0.5, NaH2PO4 1.25, NaHCO326, glucose 20). Horizontal slices containing VTA (250 μM) were cut using a tissue slicer (VT1000, Leica), then transferred to a holding chamber filled with oxygenated artificial CSF (aCSF; in mM: NaCl 126, KCl 3, MgCl2 1.5, CaCl2 2.4, NaH2PO4 1.2, NaHCO3 26, glucose 11) at room temperature.
Confocal experiments used 2- to-3-month-old female TH-Cre rats. Two to three weeks after bilateral injection of a GCaMP6f construct into the VTA, rats were anesthetized by isoflurane and decapitated. Horizontal VTA slices (230 μM) were cut in a solution containing (in mM) NMDG 93, KCl 2.5, MgSO4 10, CaCl2 0.5, NaH2PO4 1.2, NaHCO3 30, glucose 25, HEPES 20, sodium ascorbate 5, sodium pyruvate 3, thiourea 2; PH 7.2–7.4. Slices were first incubated in this solution at 34°C for 10–15 min and then transferred to room temperature aCSF until imaging.

Electrophysiology

A slice was transferred to a recording chamber and immersed in heated (32°C), flowing (2 ml/min), oxygenated aCSF. Slices were visualized with an upright microscope equipped with differential interference contrast (DIC) optics (BX51WI, Olympus). Recorded neurons were in lateral VTA, medial to the terminal nucleus of the accessory optic tract and anterior to the third cranial nerve. Whole-cell voltage-clamp recordings were made with an Axopatch 200B (Molecular Devices) amplifier. Pipettes (3–5 MΩ) were filled with (in mM): K-gluconate 140, NaCl 2, MgCl2 1.5, HEPES 10, EGTA 0.1, Mg-ATP 4, Na2-GTP 0.3, Tris-phosphocreatine 10 (pH 7.2), 290 mOSM. Cell-attached recordings were performed with the same electrodes immediately after patch formation to identify DA neurons by electrophysiological properties; spontaneous, pacemaker firing (1–4 Hz), and action potential widths >1.5 ms (
Chieng et al., 2011
). DNQX (10 μM), D-AP5 (25 μM), picrotoxin (100 μM), and strychnine (1 μM) were present to block AMPA, NMDA, GABAA, and glycine receptors, respectively. After rupture of the membrane patch, 5 stimuli at 40 Hz (200–600 μA, 200 μs) were applied every 60 s, via a bipolar

electrode placed in anterior VTA, to evoke GABABreceptor currents. Stimulation protocols were generated and signals acquired using the WinLTP program (WinLTP, The University of Bristol, Bristol, UK) on a personal computer.

Expression of Genetically Encoded GCaMP6f and Imaging

TH-Cre rats were anesthetized with isoflurane, and AAV1.Syn.Flex.GCaMP6f.WPRE.SV40 virus (titer: ~1012 genomes/ml, 500 nl each site, UPENN Vector Core) was injected bilaterally into VTA (from bregma, A/P: −5.4 mm; M/L: ± 0.7 mm; D/V: −8.0 mm), using a micro pump (UltraMicroPump, WPI, 100 nl/min) and Hamilton syringe (1701RN) with hypodermic tubing (30 gauge). The tubing was left in place for 5 min after completion of the injection.

Two to three weeks after surgery, GCaMP6f fluorescence was observed using a confocal microscope (LSM710, Zeiss, W Plan-Apochromat 20× objective) and 488 nm laser in slices in aCSF. Images were collected at 0.5 Hz. After 5–10 min of baseline, slices were exposed to drug for 20 min. Image stacks were processed and analyzed offline with ImageJ (NIH). When observed, a StackReg plug-in was used to correct x-y drift. Regions of interests were defined over cell bodies. The time-lapse signal was expressed as $\Delta F/F0$, where $F0$ is the baseline fluorescence and $\Delta F = F - F0$. The frequency of calcium oscillations was determined by fast Fourier transformation (FFT) of the signal. Half-width was measured using Event Detection in Clampfit 9 (Molecular Devices). Cross correlation of the calcium signals was performed in Clampfit 9, and the peak correlation coefficient was chosen within a ±10-s window.

Measurement of DA Transients In Vivo Using FSCV

Male Sprague-Dawley rats purchased with implanted jugular vein catheters (n = 9, 300–350 g; Charles Rivers) were anesthetized with isoflurane. A guide cannula (Bioanalytical Systems) was positioned above the NAc shell (from bregma, A/P: +1.7 mm, M/L: +0.8 mm, D/V: −2.5 mm), and a combination bipolar stimulating electrode/steel guide cannula (26 gauge; Plastics One) was implanted ipsilaterally targeting the VTA (from bregma, A/P: 5.4 mm, M/L: 1.2 mm, D/V: 8.2 mm). An Ag/AgCl reference electrode was placed in contralateral cortex. All components were permanently affixed with dental cement. Animals were allowed 3–5 days for recovery.

FSCV was used to monitor DA release using a triangular input waveform (0.4 V to 1.3 V, 400 V/s) at 10 Hz, applied to a glass-encased carbon fiber electrode lowered into the NAc. Endogenous and electrically evoked (biphasic; 2 ms/phase, 24 pulse, 60 Hz, 125 µA) DA release was recorded prior to the experimental session to verify electrode performance and create a training set for principal component regression (PCR) to extract the DA component from the raw voltammetric data. Rapid DA concentration changes (i.e., DA "transients") were analyzed using Mini Analysis (Synaptosoft) and custom written MATLAB (The MathWorks) routines to determine drug effects on transient frequency and amplitude (
Cheer et al., 2007
).

Intra-VTA infusions of vehicle, rimonabant, or tetrahydrolipstatin (THL) occurred in the experimental chamber using a microprocessor-controlled infusion pump (Harvard Apparatus) at a flow rate of 0.5 µl/min. An infusion needle was inserted through a guide cannula ending 1 mm above the tip of the bipolar stimulating electrode (Plastics One); the needle was cut to extend 1 mm beyond the cannula tip. A within-subjects design was used where animals first received microinfusions of vehicle, followed by 3 mg/kg intravenous cocaine. A second infusion of rimonabant or THL occurred after the baseline frequency of DA transients was reestablished (~45–60 min) and preceded a second cocaine injection (3 mg/kg).

Drugs

For in vitro experiments, drugs were first dissolved in vehicle to make 1,000- or 10,000-fold concentrated stock solutions. The drugs were then applied to the slices by diluting the stock solution in the perfusion reservoir. DNQX, D-(−)-2-amino-5-phosphonopentanoic acid (D-AP5), GR55562, HEAT, JNJ16259685, MTEP, AM 251, THL, phenylephrine, sumatriptan, and U73122 were purchased from Tocris. Picrotoxin was purchased from Sigma. AAV1.Syn.Flex.GCaMP6f was purchased from the University of Pennsylvania Vector Core. 5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-[(E)-piperidinoiminomethyl]-1H-pyrazole (PIMSR1) was provided by Dr. Herbert H. Seltzman at Research Triangle Institute (NIDA, Raleigh, NC; RTI International). For FSCV experiments, cocaine (Sigma-Aldrich) was dissolved in saline, rimonabant (SR141716A; RTI), and THL (Sigma-Aldrich) were freshly suspended in a 1:1:18 ratio of ethanol, emulphor (Alkamuls EL-620; Rhodia), and saline (0.9%).

Data Analysis

Group data are presented as the mean ± SEM in all cases. All statistical tests, including t tests, one-way ANOVAs, and K-S tests were performed using GraphPad Prism 5 (GraphPad Software). Post hoc analysis (Bonferroni test) was performed when an ANOVA yielded a significant ($p < 0.05$) main effect.

Author Contributions

H.W., C.R.L., and J.F.C. conceived the study. H.W. collected and analyzed most of the electrophysiological and calcium imaging data. T.T. collected some of the electrophysiological data. D.P.C. collected and analyzed all of the in vivo data. All authors contributed to writing the manuscript.

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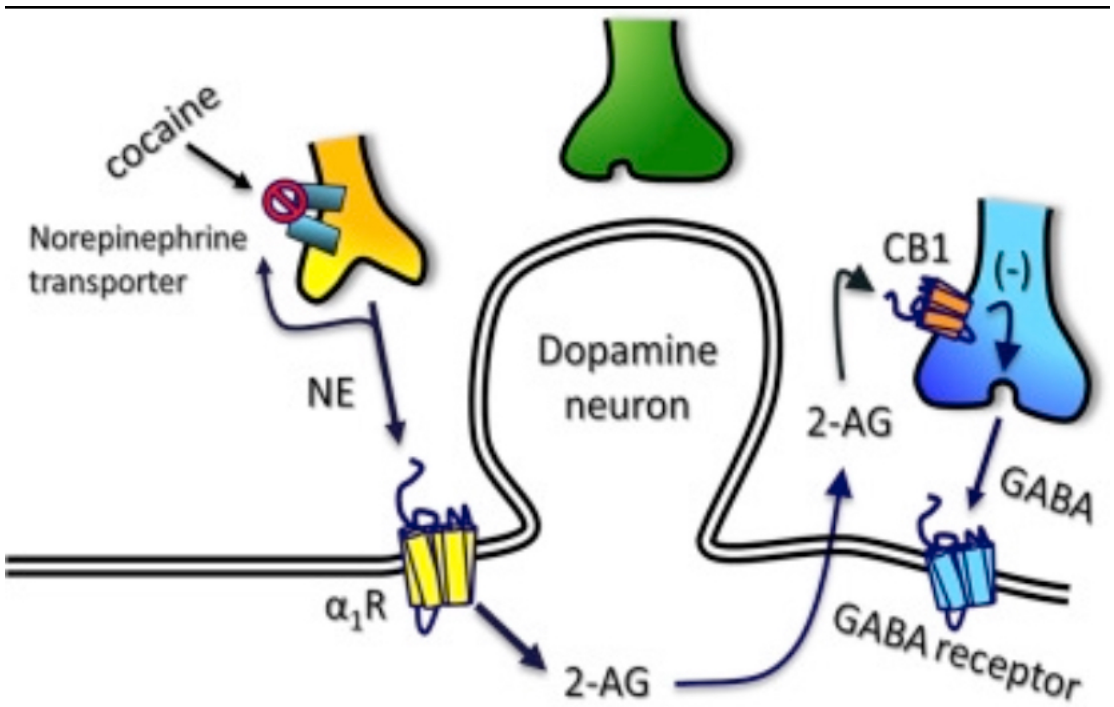
Movie S1. Cocaine-Induced Changes in Calcium Levels in VTA DA Neurons Measured by Confocal Imaging of GCaMP6f In Vitro, Related to Figure 6. Horizontal VTA slices were cut 18 days after AAV1.Syn.Flex.GCaMP6f injection. Confocal images were taken at 0.5 Hz with a 20×

objective. Spontaneous calcium changes were monitored for 5 min before cocaine was applied for 20 min (indicated in movie). Image stacks were processed offline using the StackReg plug-in of ImageJ, and corrected for x-y drift. The video rate is 40× faster than actual time. Neurons whose calcium signals were decreased by cocaine are marked by blue arrow heads, whereas neurons showing initiation of slow calcium oscillation after cocaine application are marked by yellow arrow heads.



A link between cocaine and endocannabinoids in brain reward

- Inhibition of norepinephrine (NE) transporter activates α_1 receptors (α_1R) to stimulate endocannabinoid (2-AG) production.
- 2-AG activates cannabinoid CB1 receptors to reduce release of inhibitory neurotransmitter (γ-aminobutyric acid, GABA) onto dopamine neurons.
- GABA release inhibition excites dopamine neurons to increase dopamine function.

Modulation Of The Endo-Cannabinoid System: Therapeutic Potential Against Cocaine Dependence

Gianluigi Tanda

Abstract

Dependence on cocaine is still a main unresolved medical and social concern, and in spite of research efforts, no pharmacological therapy against cocaine dependence is yet available. Re- cent studies have shown that the endocannabinoid system participates in specific stages and aspects of drug dependence in general, and some of this evidence suggests an involvement of the cannabinoid system in cocaine effects. For example, cocaine administration has been shown to alter brain endocannabinoid levels, and the endocannabinoid system has been in- volved in long-term modifications of brain processes that might play a role in neuro/behavioral effects of psychostimulant drugs like cocaine. Human studies show that marijuana dependence is frequently associated with cocaine dependence, and that the cannabinoid receptor CNR1 gene polymorphism might be related to cocaine addiction. This article will review the main papers in the field showing how a modulation of different components of the cannabinoid sys- tem might interact with some of the neurobiological/behavioral effects of cocaine related to its reinforcing effects, evaluated in preclinical models or in clinical settings. The goal of this review will be to provide insights into the complex picture of cocaine abuse and addiction, and to extrapolate from such endocannabinoid-cocaine interactions useful information to test the therapeutic potential of cannabinoid ligands and endocannabinoid-level enhancers against co- caine dependence for future preclinical/clinical trials.

Keywords: cannabinoids, cocaine, addiction, behavior

1. INTRODUCTION

A number of studies suggest that the cannabinoid system interacts with cocaine's effects at dif- ferent levels in the brain. For example, both CB1 agonists and cocaine selectively increase dopamine neurotransmission in the nucleus accumbens shell (1, 2, 3, 4), a brain area believed to play a pivotal role in the addictive effects of drugs (5). It is also interesting to note that co- caine administration results in increased levels of anandamide, one of the endogenous ligands for cannabinoid receptors in the brain (6, 7), and that blockade of CB1 cannabinoid receptors attenuates a dopamine signal triggered by cocaine in the NAc (8).

The cannabinoid system has also been implicated in brain processes related to potentiation or depression of the strength of synaptic signals (9, 10, 11, 12). These so-called processes of long term potentiation and long-term depression activated by cannabinoid neurotransmission might play a significant role in those neurobiological adaptations which are believed to take an im- portant part in the road leading from drug use to drug abuse and addiction (13, 14, 15). More- over, cannabinoid receptors are located in brain areas that have been assigned a key role in brain functions linked to the development of drug-dependence (16). Plant-derived and endoge- nous cannabinoid ligands have been suggested to affect neurotransmitter transporter functions (17, 18, 19, 20, 21, 22), which are also pharmacological targets for cocaine (23, 24). Finally, it is interesting to note that cocaine dependence is often diagnosed in the presence of other drug dependencies (25), and marijuana dependence is second only to alcohol dependence as the most frequent comorbid dependence in cocaine addicts (25, 26).

1.1 Overview on cocaine abuse and addiction

Cocaine abuse and addiction are widespread throughout the world, and these phenomena are driven by the ability of this drug to produce reinforcing effects by interacting with specific sites in the central nervous system (23). Cocaine binds to specific transport sites on the cell mem- branes of neurons that release dopamine, serotonin or noradrenaline. These transport sites have the physiological function of removing neurotransmitters from the extracellular space after their release from nerve terminals (24). Binding of cocaine to the neurotransmitters' transport sites results in blockade of the transport, increased availability of neurotransmitters for binding to their respective receptors, and ultimately increased neurotransmission (23, 27).

Although cocaine also binds and blocks the transporters for serotonin and noradrenaline (24), blockade of the dopamine transporter (DAT) appears to be the pharmacological target of co- caine mainly related to its reinforcing and other behavioral effects (23, 27). Thus, increased availability of dopamine for dopamine receptors is believed to mediate most of the reinforcing actions of cocaine, leading to its abuse liability (1, 3, 23, 27). However, cocaine abuse and ad- diction are processes that go well beyond the pharmacological effects of cocaine on dopamine neurotransmission, so that many other factors, genetic and environmental for example, have been suggested to play a role in the complex of actions that lead from cocaine use to its abuse and addiction (28, 29, 30, 31).

There is no specific pharmacological therapy available against cocaine's addictive effects, but researchers in the last decades have made progresses in the understanding of the neurobiology underlying cocaine's actions (29, 30). Today, different aspects of cocaine abuse and addiction in humans can be modeled and studied in experimental animals. These animal models have provided valuable results leading to a better understanding of the different factors contributing to cocaine effects (32). Moreover, the models have been invaluable in the study of newly syn- thesized compounds acting over different neuronal systems that might become candidates for development as cocaine abuse medication (29, 30).

1.2 Overview on the brain endocannabinoid system

The important discoveries in the last two decades in the cannabinoid field have provided useful information about the physiological functions of the endocannabinoid system in the brain and periphery, and the involvement of this system in the etiology and therapy of many human dis- eases (33, 34, 35). During the last decade researchers have provided evidence for the existence of at least two cannabinoid receptors, CB1 and CB2 (36, 37, 38, 39) and we have now avail- able different selective synthetic agonists and antagonists for both CB1 and CB2 receptors (40, 41), other than classic, plant-derived cannabinoid agonists like delta-9-THC, the active ingre- dient in marijuana. Although the existence of novel cannabinoid receptors has been suggested in different studies (42, 43, 44), definitive proof of the existence of these cannabinoid receptors has yet to be shown. Several different endogenous ligands for CB1 and CB2 receptors have been found, such as anandamide, 2AG, noladin-ether (45, 46, 47, 48). At variance with classical neurotransmitters, these endocannabinoids are not stored in vesicles in nerve endings, but are released "on de- mand" by specific changes occurring in neurons which promote the function of specific en- zymes that produce anandamide or 2-AG from membrane-constituent lipids (49, 50, 51, 52). We now have evidence that endogenous cannabinoids released "on demand" can function as retrograde messengers in the synapse (53). Thus, activation of cannabinoid receptors might change the electric equilibrium of the post-synaptic membranes with important modifications for cell-depolarization, neuronal firing, and release of classic neurotransmitters, which ulti- mately results in a modification of brain neurotransmission (10, 11).

Some of the neurobiological effects of endocannabinoids have been demonstrated to be long- lasting, leading to the so-called processes of long-term potentiation and long-term depression of the synaptic transmission strength (11, 12). It is interesting to note that these long-term ef- fects are in spite of the very brief half life of endogenous cannabinoid ligands in the extracellu- lar space (49, 54, 55).

Anandamide and 2-AG are taken-up into the cell by a not completely defined and still debated mechanism/s: passive diffusion or facilitated transport across the plasma membrane (55, 56, 57, 58, 59). Anandamide is then metabolized into the cell by an intracellular enzyme, the fatty acid amide hydrolase (FAAH) (60, 61), the blockade of which has been shown to prolong the behavioral/neurobiological effects of anandamide (4, 61, 62, 63). In contrast, 2-AG is metabo- lized through the mono glyceril lipase enzyme (54). In the last few years several pharmacologi- cal tools have been discovered that alter the physiological equilibrium of the endocannabinoid system by acting on the metabolic pathways of the endogenous ligands. The result of this mod- ulation is that blockade of endocannabinoid metabolism increases levels of endocannabinoids in specific brain areas in which there is a preexisting cannabinoid tone or where endogenous cannabinoids are released on demand triggered by specific neuronal signals (34, 61). These highly-specific effects contrast with the effects of exogenously administered cannabinoid ago- nists, which diffuse widely throughout the brain leading to a generalized and contemporaneous activation of multiple brain areas.

It is believed that in humans, cannabinoid receptor agonists, like delta-9-THC, activate cannabinoid CB1 receptors, producing reinforcing effects which are the basis for marijuana abuse (2, 64). CB1 receptors are also involved in other brain mechanisms and functions associ- ated with pathologies in which a cannabinoid intervention might have a therapeutic value (33, 34, 65, 66). Unfortunately, there is no cannabinoid agonist drug available yet that can selective- ly produce therapeutic effects without having undesired psychotropic, reinforcement-related effects. However, pharmacological tools are now available that can modulate the activity of endogenous cannabinoid ligands in selected brain areas, thereby having therapeutic value (34) without the harmful reinforcing effects produced by delta-9-THC-like drugs with the wide- spread action. Indeed, interesting actions of such cannabinoid modulators have been already described in preclinical models of anxiety (61, 67) and depression (68), and preclinical tests so far have also shown negative results in procedures involving models of drug abuse (4, 68, 69).

2. Effects of cannabinoid drugs or endocannabinoid modulators in preclinical models of cocaine's behavioral and reinforcing effects

2.1 Cannabinoid interactions with cocaine-induced stimulation of motor activity and behavioral sensitization

2.1.1 Effects after acute administration of cocaine Psychostimulants, like cocaine, have the abil- ity to influence neuronal activity, which results in stimulation of behavioral activity. Stimula- tion of locomotor activity induced by cocaine and other psychostimulants has been extensively studied (see, for example citations 70, and 71). Administration of psychostimulants in rodents induces a dose-dependent increase in behavioral activation, which depends on the activation of selected brain striatal areas (72, 73, 74, 75, 76), and that is

accompanied by stimulation of DA neurotransmission (3, 77, 78, 79). Although this behavior is not a direct correlate of the rein- forcing effects of cocaine, the fact that it can be obtained by injecting cocaine in the NAc (72, 76, 77), and that NAc DA transmission is involved in different aspects of drug abuse and ad- diction (5, 80, 81), makes monitoring of locomotor activation an easy preclinical target to test drugs that can interfere with the ability of cocaine to stimulate DA neurotransmission and po- tentially attenuate cocaine-induced reinforcement (82).

Docannabinoidsaltertheacuteeffectsofcocaineonstimulationofmotoractivities? Pryor and colleagues, 1978 (83), described the interaction between cocaine and delta-9-THC on mo- tor activity, measured as photo-beam interruptions in rats, as one of mutual antagonism depen- dent on the combination of doses tested. The antagonism was attenuated but still evident after sub-chronic administration of delta-9-THC. In agreement with these data, Ferrari et al., 1999 (84), showed that HU210, a synthetic agonist for CB1 receptors, dose dependently reduced the locomotor activity induced by cocaine, 15 mg/kg i.p. in rats. However, sub-chronic administra- tion of HU210 was not able to modify cocaine-induced locomotion at variance with the previ- ous report. It should be noted that a cannabinoid antagonist was not available in 1978, and that Ferrari et al., 1999 (84), did not test whether a CB1 receptor antagonist could interfere with HU210 actions on cocaine effects. Because of the lack of direct pharmacological involvement of CB1 receptors in cocaine effects, Przegalinski et al., 2005 (85), tried to address this issue in a more recent report. In that study the authors showed that the CB1 receptor agonist WIN 55,212-2, but not its less active enantiomer WIN 55,212-3, dose dependently (3–6 mg/kg i.p.) reduced cocaine-induced hyper-locomotion, without affecting behavior when administered alone. Rimonabant (SR 141716), a selective antagonist of CB1 receptors (86), did not reverse the effects of WIN 55,212-2 on cocaine-stimulated locomotion, suggesting a non-CB1 mediat- ed mechanism of action for WIN 55,212-2. Moreover, the effect was not attributable to unspe- cific interactions of the highly lipophilic cannabinoid agonist WIN 55,212-2 with hydrophobic sites of proteins or other cell's membrane lipid constituents, since the less active enantiomer did not show any significant effect on cocaine-induced hyper- locomotion (85). Further, the au- thors showed that the effects of WIN 55,212-2 resembled those of a serotonin 5HT3 antagonist, based on other previously published evidence (87, 88), thus indicating the possibility of an in- volvement of 5HT3 receptors in the action of cannabinoid agonists on cocaine-induced hyper- locomotion. However, other than a resemblance of effects, there was no direct pharmacological evidence for 5HT3-receptor involvement in the actions of WIN 55,212-2 on cocaine-induce hyper-locomotion (85). It should also be noted that in the report by Przegalinski et al., 2005 (85), the locomotor stimulant effects of cocaine were reduced to a certain extent, though not significantly, by Rimonabant itself, while in a recent report (8) Rimonabant significantly atten- uated cocaine-induced hyperactivity, thus suggesting the possibility that both agonist and an- tagonists of CB1 receptors can negatively modulate the locomotor stimulant effects of cocaine. Also, Rimonabant antagonized cocaine-induced hyper-locomotion in Gerbils habituated to the test cage, but not in non-habituated animals (89). The authors explained their results in terms of a cannabinoid involvement as a permissive element required in the ability of cocaine to override novelty-stimulus satiation in habituated Gerbils. However, lack of effects of Rimona- bant on acute cocaine-induced locomotor stimulation has also been reported in habituated mice and rats (90, 91).

Taken together these experiments suggest that an endocannabinoid tone might not be necessary to mediate the acute motor stimulant effects of cocaine in rodents. However, depending on dose, both cannabinoid CB1 receptor agonists and antagonists might modulate this behavioral stimulation induced by cocaine. Moreover, as shown by Cheer et al., 2007 (8), attenuation of cocaine-induced locomotor activity by a CB1 receptor antagonist has a neurochemical corre- late. In that study, Rimonabant pretreatment attenuated dopamine neurotransmission, as mea- sured by "fast-scan-cyclic voltammetry", suggesting that a cannabinoid tone might be involved in both neurochemical and behavioral processes triggered by cocaine (8). 2.1.2. Effects in cocaine-sensitized animals Psychostimulant sensitization is a phenomenon that has been extensively described in rodents. It appears as an increased effectiveness of the psy- chostimulant drugs to produce behavioral activation in animals previously exposed to single (92) or repeated (93) administration of the same drug. Cross sensitization between different drugs has also been described (94, 95).

It has been suggested that sensitization induced by drugs of abuse may be due to neuronal adaptations that play a role in the development of addictive behaviors. Behavioral sensitization is easily demonstrated in animals, and it has been hypothesized that this adaptation is involved in the transition from drug use to abuse and addiction (see citations 14, and 15, for review). Thus, behavioral sensitization has been the target of different studies searching for drugs able to impair or reduce the effects of this adaptation induced by sub-chronic/chronic administration of cocaine.

Do cannabinoids play a role in the behavioral sensitization induced by cocaine? There are only a few reports about cannabinoid interactions with development or expression of cocaine- induced behavioral sensitization. Sensitization and cross sensitization have both been reported for cannabinoid agonists and some drugs abused by humans including psychostimulant drugs (94, 95, 96, 97, 98, 99, 100). However, failure to develop cross sensitization in rats chronically treated with a cannabinoid agonist, CP 55,940, and challenged with cocaine has also been re- ported (101). In the same study, CP 55,940 failed to enhance the sensitivity to cocaine when the drugs were concurrently administered during the development phase. Also, the develop- ment of cocaine sensitization did not appear to be under control of CB1 receptors in rodents in another report (90). In this latter study, mice were treated with 20 mg/kg cocaine for 11 days in their home cages. Three days after the last cocaine injection they habituated to an open field, and then tested with cocaine (10 mg/kg). The authors reported a first set of experiments in which Rimonabant 1 mg/kg administered 30 min before the daily cocaine injection significant- ly reduced the locomotor sensitization induced by cocaine. Such effects, however, were not replicated in a subsequent set of experiments with increasing doses of cocaine (90).

In genetically modified CB1 receptor knock out mice (102), cocaine (10 mg/kg twice daily for 15 days) induced a behavioral sensitization, tested after one week withdrawal in an open field, which was indistinguishable from that induced in wild-type littermate control mice. These findings once more suggest a lack of involvement of the endocannabinoid system in the devel- opment of behavioral sensitization induced by cocaine. On the other hand, the expression of cocaine sensitization has been demonstrated to be impaired by cannabinoid CB1 receptor blockade, as shown by administration of the selective CB1 receptor antagonist Rimonabant together with cocaine five days after withdrawal

from cocaine (103). Although this interaction needs to be further investigated, results from the cited reports suggest that even if activation of cannabinoid neurotransmission through CB1 receptors might not be required for development of cocaine-induced sensitization, an endogenous cannabinoid tone might be essential for its expression.

Thus, cannabinoid antagonists might decrease the impact of a “sensitized” response to drug stimuli in subjects during abstinence. This prediction matches the results obtained by DeVries et al., 2001 (104), with administration of the cannabinoid receptor antagonist Rimonabant in cocaine abstinent rats during self-administration reinstatement tests (see below the section: “Effects of cannabinoids on animal models of extinction and reinstatement of cocaine-seeking behavior”).

2.2 Cannabinoid interactions with cocaine-induced place conditioning

The place conditioning procedure is a model of drug reinforcement and is based on an individ- ual’s behavioral choice after a certain number of pairings of drug with one environment and placebo with another. This procedure, though simple, can give an indication of the pleasurable (place preference) or aversive (place avoidance) feelings/perceptions induced by drugs during the conditioning procedure. The place-conditioning allows the study of both development and expression of aversion or preference for a place associated with a certain drug dose (see cita- tions 105, and 106 for review), with the final test performed with animals in a drug-free state. So, the ability of drugs to interfere or impair the effects of the conditioning-drug could be re- lated to a potential therapeutic value, in terms of ability to reduce or block the pleasurable feel- ings induced by the conditioning drug, or to attenuate the strength of the association between the conditioning drug and the environmental stimuli.

It has been repeatedly shown that cocaine, just like many other drugs abused by humans, sup- ports place preference at certain doses (106). On the other hand, results from place condition- ing studies with cannabinoid agonists and antagonists have been inconsistent (see citation 107 for review), and both preference and aversion induced by cannabinoid CB1 receptor agonists and antagonists have been reported under a variety of different experimental conditions and dose ranges (108, 109, 110, 111, 112, 113).

The involvement of the cannabinoid system in processes related to positive reinforcement in- duced by cocaine in the place conditioning procedure was evaluated by Chaperon et al., 1998 (91), in rats. The authors showed clear evidence that blockade of CB1 receptors by administra- tion of the selective cannabinoid CB1 receptor antagonist Rimonabant impaired the acquisition of cocaine-conditioned place preference, suggesting that the development of such conditioning was under control of a cannabinoid tone in the brain. In the same paper the authors also showed that blockade of CB1 receptors did not impair the expression of cocaine-conditioned place preference when the antagonist of CB1 receptors, Rimonabant, was administered only once, 30 min before the final test, instead of 30 min before each cocaine injection during the conditioning sessions (91). At variance with this report, results from a study by Martin et al., 2000 (102), showed that development and expression of cocaine-conditioned place preference can be established in genetically modified CB1 receptor knock-out mice. These latter results suggest no involvement of the endogenous cannabinoid systems in brain processes related to the motivational effects induced by cocaine in this procedure in genetically modified mice (102). These contradictory findings (91, 102) might be explained on the basis of species differ- ences, or developmental adaptations in the regulatory processes involved in the mediation of cocaine reinforcing effects in mice genetically deficient in CB1 cannabinoid receptors. It should be noted that Rimonabant might have inverse agonist actions on CB1 receptors (114, 115, 116), or might possess actions over pharmacological targets other than on CB1 receptors, for example on the putative CB3 receptor (42, 65). Martin and colleagues (102) did not cite the article by Chaperon et al., 1998 (91), so we do not have their comments on this issue. We also do not have information about the effects of Rimonabant administration on development and expression of cocaine-induced place preference in wild-type mice, an experiment that would allow a direct comparison with data obtained in CB1 KO mice (102).

2.3 Effects of cannabinoids on the discriminative stimulus effects of cocaine

The drug discrimination procedure has been extensively used in animal and human subjects to evaluate the specific perceptions/feelings produced by drug administration (117). It is likely that these subjective effects play an important role in the reinforcing effects of many drugs abused by humans. Indeed, many of these drugs produce euphoric or pleasurable sensations that are believed to be factors contributing to the abuse of substances (117).

The discriminative stimulus properties of cocaine have been evaluated in many differents stud- ies under different conditions and in different species, including human subjects (117, 118). However, there is a very limited set of data available in which the influence of the cannabinoid system has been evaluated in animals trained to discriminate the subjective effects of cocaine from those of placebo. Delta-9-THC did not substitute for cocaine in pigeons trained to dis- criminate cocaine from placebo, or cocaine from morphine, suggesting that the discriminative stimulus of the cannabinoid agonist and that of cocaine do not overlap (119, 120). In a more recent report (103) the cannabinoid receptor antagonist Rimonabant did not substitute for co- caine, and did not change significantly the discriminative stimulus effects of cocaine in rats. These limited findings indicate that the endogenous cannabinoid tone mediated by CB1 recep- tors does not play a role in the expression of the discriminative stimulus of cocaine. However, more detailed studies with a complete range of cannabinoid drugs (agonist, antagonist, and modulators) and doses are needed to further elucidate the role of the cannabinoid system in the subjective effects of cocaine.

2.4 Cannabinoid modulation of the effects of cocaine on thresholds for Intra Cranial Self Stimulation (ICSS)

This procedure is based on studies by Olds and Milner (121), in which rats have the opportuni- ty to learn to repeatedly press a lever in order to obtain an electrical stimulation of the medial forebrain bundle, a component of reward circuits in the brain (122). It has been repeatedly shown that most drugs of abuse are able to lower ICSS thresholds in a dose-dependent fashion (122, 123). This effect has been used as an indirect measure of their ability to produce “re- ward” through brain processes linked to the medial forebrain bundle. Thus, substances able to reduce or block the ability of drugs of abuse to lower the ICSS threshold may have therapeutic potential as treatments for drug abuse.

While the effects of cocaine in this procedure are con- sistent with its reinforcing effects in animal and human subjects, inconsistent results have been obtained with administration of cannabinoid drugs (124, 125; see also citations 106, and 126, for review). There is only limited

information available for combinations of cocaine and cannabinoids studied with the intracranial self stimulation procedure. In a study by Pradhan et al., 1978 (127), co-administration of delta-9-THC in rats resulted in an antagonism of the ef- fects of cocaine, while in experiments in which delta-9-THC was administered 80 min before cocaine, the effects of cocaine were unaffected by the cannabinoid agonist. In agreement with this study, in a more recent report (128) administration of the synthetic cannabinoid agonist WIN 55,212-2 reduced the effects of cocaine on ICSS thresholds. In the same study, adminis- tration of the CB1 receptor antagonist, Rimonabant, antagonized the effects of WIN 55-212,2 on cocaine. Also, Rimonabant administration did not affect cocaine actions on this procedure, though only very low doses of the CB1 antagonist were tested (128).

These above cited reports suggest that cannabinoid agonists might have some potential activity against cocaine reinforcing effects in rodents, however, the mechanism/s for these interactions are not clear. Drugs of abuse and intra cranial self stimulation produce reinforcing effects through the same reward circuitry (122), and also increase dopamine neurotransmission in a dopaminergic reward-related area of such circuitry (1, 2, 3, 78, 129, 130) that has been related to their reinforcing effects (5, 79, 80). Thus, it is not apparently clear why cocaine and cannabi- noid CB1 receptor agonists, which share the ability to stimulate the brain reward circuitry, may have different and antagonistic effects in this procedure. However, the fact that in rodents cannabinoid agonists do not consistently produce reinforcing effects, but do so only under cer- tain experimental conditions (108, 124, 131, 132, 133, 134; see also 107, 126, and 135), sug- gests a cautious approach to the outcome of experiments involving reinforcing effects of cannabinoids in these species as compared to the well known consistent reinforcing effects of marijuana in human subjects.

2.5 Effects of cannabinoids on cocaine-maintained self-administration behavior

Although the most direct outcome of this procedure is to predict reinforcing effects and abuse liability in humans for drugs that maintain self-administration behavior in experimental ani- mals, many other aspects of this behavioral procedure can contribute to a better understanding of the behavioral effects of drugs and drug-related cues, their related neurobiology and poten- tial therapeutic interventions (136, 137).

As a drug that is widely abused, cocaine consistently maintains intravenous self-administration behavior across a range of doses in a variety of different species (see, for example, citations 138, and 139). Although the reinforcing effects obtained by administration of plant derived cannabinoids in humans were described in books thousands of years ago, cannabinoid agonists do not consistently maintain self-administration behavior in experimental animals (see cita- tions 107, 126, and 135 for review).

Preclinical studies have shown that pharmacological alterations of the endocannabinoid recep- tor system might interfere with the ability of cocaine to maintain self-administration behavior. In Long Evans rats, administration of the cannabinoid agonist WIN 55-212,2 reduced the aver- age number of intravenous self-injections of different doses of cocaine under a fixed-ratio 1 schedule, suggesting a potentiation of cocaine effects (140). In the same report, the selective antagonist of CB1 receptors Rimonabant antagonized the effects of WIN 55-212,2 on cocaine self-administration behavior. Rimonabant, however, did not affect the behavior maintained by cocaine when administered alone (140). In agreement with this report, Rimonabant pretreat- ment did not show any effect on cocaine self-administration under fixed-ratio schedules of be- havior in squirrel monkeys (63), an effect that has been recently replicated and confirmed (141, 142). In addition, Rimonabant did not affect the number of cocaine self-injections in Wistar rats studied under a fixed-ratio 5 schedule of drug delivery (103, 104). In agreement with the previous experiments in rats and primates, pre-treating C57Bl/6J mice with Rimonabant before single one-day sessions of cocaine self-administration affected neither the number of nose- pokes maintained nor the total cocaine intake during the 30-min session (90). In contrast the effects of genetic deletion of CB1 receptors have not been entirely consistent, and can differ with the effects of administering the pharmacological antagonist of CB1 receptors, Rimona- bant. For example, using the single one-day cocaine self-administration procedure mutant cannabinoid CB1 receptor knock-out mice failed to self-administer morphine, but were still able to self-administer cocaine, suggesting that the endocannabinoid system was a substrate for mediating the reinforcing effects of opioids but not those of cocaine (143). At variance with these findings, a more recent report, using the same mutant cannabinoid CB1 receptor knock- out mice as subjects, showed a reduced number of mice reaching the acquisition criteria, which was also obtained within a lager number of sessions, and a significantly reduced reinforcing efficacy of cocaine (measured as the effort required to obtain a cocaine infusion) as compared to wild-type littermate control mice (144). The authors explain their results, contrasting with those of Cossu et al., 2001 (143), in terms of different experimental conditions; freely moving mice chronically receiving cocaine self-infusions in daily sessions (144) versus mice with re- strained mobility receiving cocaine during a single session (143).

Soria and colleagues (144) confirmed their data in mutant CB1 receptor knock-out mice study- ing the effects of pretreatments with the antagonist of CB1 receptors Rimonabant in wild-type mice on cocaine self-administration under a progressive-ratio schedule. They found that Ri- monabant dose-dependently reduced the breaking point achieved by wild-type mice under the progressive-ratio schedule of cocaine self-administration, suggesting that also in normal con- trol mice the endocannabinoid system was a substrate for the reinforcing effects of cocaine (144). It is interesting to note that in this report the authors excluded any involvement of learn- ing/memory processes in the effects of cocaine in mice lacking the CB1 receptors because the same mice successfully acquired an operant response maintained by natural reinforcers (food and water). Also, the reduced efficacy of cocaine as a reinforcer was not the result of a dimin- ished ability of cocaine to stimulate dopamine neurotransmission, measured by intra-cerebral microdialysis in limbic areas of mutant mice as compared to their littermate controls (144). These latter results point toward a more complicated and central involvement of the endo- cannabinoid system in the addictive effects of cocaine.

As neither deletion nor blockade of CB1 receptors impairs cocaine's ability to stimulate DA neurotransmission, it seems that the effects of modulation of the endocannabinoid system on cocaine self administration may be indirect. In a recent report using Sprague Dawley rats, prior exposure to delta-9-THC did not affect the acquisition and subsequent rates of cocaine self- administration under a fixed-ratio 1 schedule of injection, but did reduce the number of "co- caine-seeking" responses under a progressive-ratio schedule (145). Thus the sensitivity to delta-9-THC exposure may vary with behavioral procedure. Indeed, it has been already report- ed that changes in reinforcing

efficacy of cocaine are hard to detect using fixed-ratio schedules of cocaine self-administration (146). In the report by Panlilio and colleagues (145), delta-9-THC pre-exposure also decreased both the number of entries in the center-zone of an open- field arena, and the time spent in the lighted compartment of the dark-light box (two different predictors of an anxiety response) in cocaine-treated rats. The authors suggested that the re- duced effectiveness of cocaine as a reinforcer after delta-9-THC pre-exposure may be because the previous exposure to the cannabinoid agonist enhanced the aversive/anxiogenic effects of cocaine (145).

The contrasting results between species and schedules of drug delivery together suggest that the endocannabinoid system might impair other brain processes related to cocaine self admin- istration which might in turn reduce the ability to detect the reinforcing effects of cocaine. Cer- tainly, a better understanding and clinical prediction of the influence of the endocannabinoid system on the reinforcing effects of cocaine will come from studies of the effects of cannabi- noid agonists and antagonists under a variety of procedures (including progressive-ratio sched- ules) and with a variety of cannabinoid exposure procedures in primates.

2.6 Effects of cannabinoids on animal models of extinction and reinstatement of cocaine- seeking behavior

One of the major obstacles to successfully achieving withdrawal or abstinence from drug use stems from the difficulty of finding effective strategies against relapse to drug use that is thought to result from craving for the abused substance. Other than behavioral therapy, poten- tial pharmacological support might alleviate craving in abstinent individuals, thus reducing the likelihood of relapse. Although the validity of preclinical models of relapse to drug-seeking and drug-taking behaviors is still under discussion (147, 148, 149, 150), these procedures might give some useful information about which drugs and which brain areas might interfere with the processes that underlie relapse.

In the last years, as it has become increasingly clear that the endocannabinoid system plays a role in different aspects related to substance abuse, researchers have explored the involvement of the cannabinoid system in the neurobiology of relapse to drug-seeking (see citation 151 for review). In rats, when cocaine self-administration behavior was extinguished by substituting saline for cocaine injections (152), experimenter administration of increasing doses of delta-9- THC, 3 hours after withdrawal, failed to induce/reinstate cocaine-seeking behavior. However, administration of HU210, a synthetic cannabinoid agonist which has a higher affinity and greater intrinsic activity than delta-9-THC at CB1 receptors, provoked relapse to cocaine-seek- ing after a prolonged (14 days) withdrawal from cocaine (104). In addition to differences in treatment, the different outcome of these reports could be due to differences in abstinence strength, because in the first and second reports rats were abstinent from cocaine only for three hours (152) and 14 days (104), respectively. These different conditions might suggest time-re- lated adaptations of the brain systems involved in cocaine-seeking behaviors (153), with no influence of the endocannabinoid system during the early stages of withdrawal from cocaine. On the other hand, when the selective antagonists of cannabinoid CB1 receptors Rimonabant and AM251 were administered alone they did not reinstate cocaine-seeking behavior (104, 154), but both drugs reduced or inhibited cocaine-seeking behavior evoked by priming injec- tions of cocaine (104, 154). It is also interesting to note that Rimonabant reduced cocaine seek- ing behavior induced by cocaine associated cues, but not stress-induced reinstatement (104), suggesting the involvement of the endocannabinoid system only in some specific processes or pathways related to cocaine-seeking behavior. To this end, it is also interesting to note that AM251 did not block the DA-stimulating effects of cocaine in the NAc, but it attenuated the effects of cocaine on glutamate levels in the NAc, which might be a better neurochemical cor- relate of cocaine-seeking behavior (154).

3. Human studies

Though not overlapping with the purpose of this review, the “gateway drug” hypothesis of marijuana use is an interesting example of a link or association between cocaine dependence and cannabinoid agonists. Following this hypothesis, marijuana consumption could serve as a gateway drug for subsequent use and abuse of other illicit substances in a progression that can lead towards dependence on heroin, cocaine or other psychostimulants (155, 156, 157, 158). There is still an ample debate about the factors studied and taken into consideration, and the data obtained which support this theory (see for example citations 159, and 160). It is interest- ing to note, however, that independently from the factor/s that link marijuana use to subsequent abuse of other illegal substances, there are also studies that show a very high prevalence of marijuana use and dependence in cocaine dependent individuals (26, 161, 162, 163). Comor- bidity of cocaine dependence with other drug dependencies is also very common (25). Thus the occurrence of cannabis dependence in cocaine dependent subjects might suggest that indi- vidual vulnerability for a certain drug can be extended to other drugs (160), but also that com- mon risk factors (genetic, environmental, etc.) might jointly influence the individual drug lia- bility and the links between cannabis and other drug dependencies (163).

With regard to genetic risk factors, it is interesting to note that a human genetic variant of the cannabinoid receptor gene CNR1 has been associated with susceptibility of drug dependence in a non-Hispanic, Caucasian population (164). The association was stronger with intravenous drug use, and was greatest for cocaine, amphetamine, and marijuana dependence. Non-signifi- cant association data have been instead obtained in replication of the original findings by Comings and colleagues (165, 166). In a recent paper (31) these contrasting data are discussed as the result of the poor definition of the cannabinoid receptor CNR1 gene’s structure, regulation and variation. In the same report, indeed, the authors have shown an improved definition of the human cannabinoid receptor CNR1 gene’s locus and variants (31), and it has been also shown that haplotypes toward the 5′ end of the cannabinoid receptor CNR1 gene’s exons and introns differentiate between non-drug users and drug-abusers of European-American, African-Ameri- can, and Japanese subjects. Also, a recent report by Ballon and colleagues, 2006 (167), sup- ports the association of the cannabinoid receptor CNR1 gene polymorphism with predisposi- tion to cocaine dependence in an African-Caribbean population.

Another interesting association, from a therapeutic perspective, has been evaluated studying the relationship between marijuana use during cocaine abstinence and relapse to cocaine use. In a study by Labigalini et al., 1999 (168), the authors describe a clinical observation based on spontaneous accounts by Brazilian cocaine-crack abusers, undergoing their first psychiatric assessment, reporting use of cannabis in order to get relief from cocaine-withdrawal symp- toms. From a sample of 25 subjects, 68 percent ceased to use crack-cocaine during the study, which lasted 9 months. The small sample and the short period of time suggest a cautious ap- proach to the results outcome; further studies are warranted. A more recent study (169) in New York used a much larger sample of cocaine addicts (144 subjects), and examined

whether cannabis use after discharge from inpatient treatment could affect relapse to cocaine use. Data from this study showed that about a third of the subjects used cannabis after discharge and that cannabis use substantially increased the risk of relapse to cocaine use, significantly reduced the achievement of sustained remission, and significantly increased relapse to cocaine use after sustained remission (169). In agreement with this latter report is a preclinical investigation (de- scribed above, section 2.6) showing cannabinoid-induced reinstatement of cocaine seeking be- havior in rodents (104).

4. Summary and concluding remarks

Most of the scientific articles reviewed in the present manuscript have described studies of cannabinoid CB1 receptor agonists and antagonists tested against cocaine effects in preclinical models thought to be predictive of cocaine abuse. These studies have provided interesting re- sults, especially for the ability of cannabinoid antagonists, and Rimonabant in particular, to significantly counteract some of the reinforcing actions of cocaine (104, 143). Collectively, the studies suggest that a cannabinoid tone, impaired by cannabinoid antagonist administration, is indeed involved in many of the reinforcing effects of cocaine which are believed to be respon- sible for cocaine abuse and addiction. On the other hand, there are no studies available show- ing interactions of drugs acting as cannabinoid levels modulators/ enhancers on cocaine-induced behaviors. These drugs affecting directly the endogenous cannabinoid tone could inter- fere with cocaine effects in these preclinical procedures and could substantially increase our knowledge about the cannabinoid-neurobiology related to cocaine dependence.

Suggestions about possible genetic predisposition/vulnerability to cocaine dependence from human studies due to variants of the cannabinoid receptor CNR1 gene have given more strength to the link between endocannabinoids and cocaine. Due to the widespread distribution of cannabinoid receptors in the brain, and their abundance in brain areas playing pivotal roles in drug abuse and addiction, the different expression and regulation of cannabinoid receptors induced by genetic differences (31) might be an important factor in the predisposition or vul- nerability to drug dependence. For this reason, the potential to directly interact with endo- cannabinoid tone in selected brain areas, an effect that can be obtained with endocannabinoid uptake inhibitors or metabolism blockers (4, 60, 62)(as shown also in genetically modified mice, 170), as compared to widespread actions of cannabinoid receptors agonists/antagonists, should be one of the next challenges in the research for medications able to counteract the abuse- and dependence-related behavioral/neurobiological effects of cocaine.

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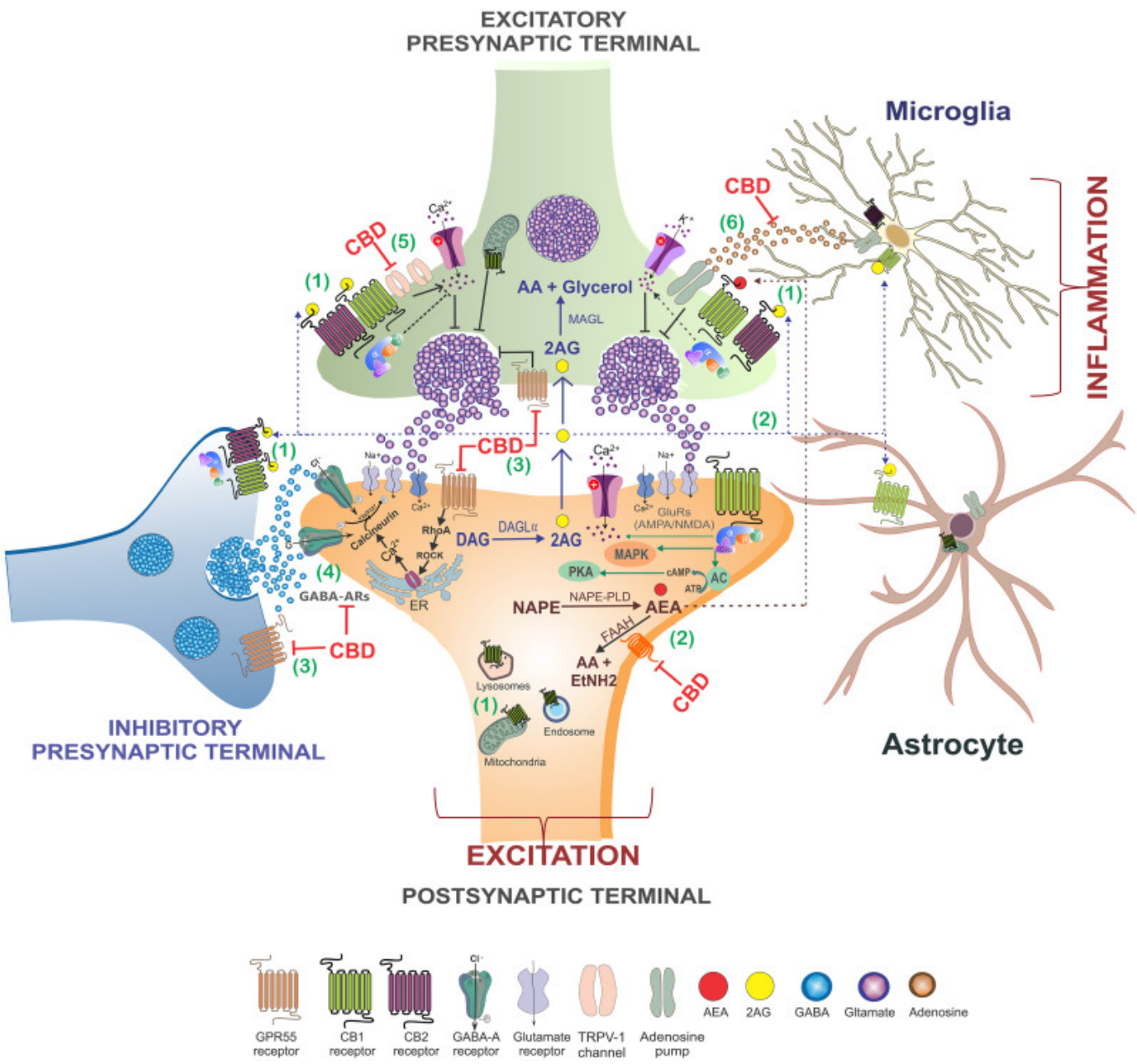

Use of Cannabidiol in the Treatment of Epilepsy: Efficacy and Security in Clinical Trials

Serena Silvestro, Santa Mammana, Eugenio Cavalli, Placido Bramanti, and Emanuela Mazzon* Raffaele Capasso, Academic Editor and Lorenzo Di Cesare Mannelli, Academic Editor

Abstract

Cannabidiol (CBD) is one of the cannabinoids with non-psychotropic action, extracted from Cannabis sativa. CBD is a terpenophenol and it has received a great scientific interest thanks to its medical applications. This compound showed efficacy as anti-seizure, antipsychotic, neu- roprotective, antidepressant and anxiolytic. The neuroprotective activity appears linked to its excellent anti-inflammatory and antioxidant properties. The purpose of this paper is to evaluate the use of CBD, in addition to common anti-epileptic drugs, in the severe treatment-resistant epilepsy through an overview of recent literature and clinical trials aimed to study the effects of the CBD treatment in different forms of epilepsy. The results of scientific studies obtained so far the use of CBD in clinical applications could represent hope for patients who are resis- tant to all conventional anti-epileptic drugs.

Keywords: cannabidiol, treatment-resistant epilepsy, clinical trials

1. Introduction

Cannabis sativa L. is an ancient medicinal plant wherefrom over 100 cannabinoids are extract- ed [1]. Among them, the most studied are Δ9–tetrahydrocannabinol (Δ9–THC), a psychoactive compound, and the CBD, a non-psychotropic phytocannabinoid [2]. CBD is a cyclohexene which is substituted in position 1 by a methyl group, by a 2,6-dihydroxy-4-pentylphenyl group at position 3, and with a prop-1-en-2-yl group at position 4 (Figure 1). Most cannabinoids exert their action by interacting with cannabinoid receptors, but CBD shows a low affinity for these receptors. Nevertheless, it affects the activity of other receptors such as serotonin receptors [5- HT], opioid receptors [ORs], and non-endocannabinoid G protein-coupled receptors (GPCRs) [3] and other targets (ion channels and enzymes).

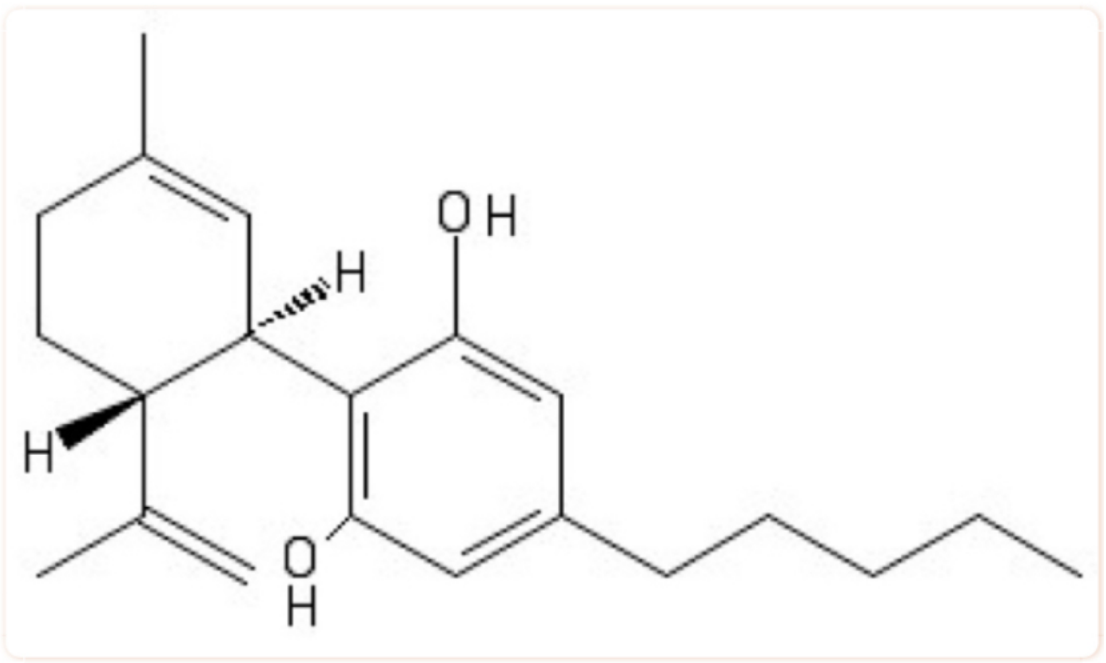

Figure 1

Structure of CBD.

In recent years, the scientific community has shown interest in this compound due to its good safety profile and neuroprotective properties [4] in several neurodegenerative diseases, includ- ing Amyotrophic Lateral Sclerosis [5], Parkinson's [6,7], Huntington's [8] and Alzheimer's diseases [9,10,11]. This neuroprotective action is due to its anti-inflammatory [12,13] and an- tioxidant [14,15] properties. CBD shows anti-inflammatory properties in several experimental studies, modulating some pro-inflammatory cytokines such as interleukin-1β (IL-1β) [16], interleukin-6 (IL-6) [17,18] and tumor necrosis factor α (TNF-α) [16,18], as well as regulation of cell cycle and immune cells' functions [19].

Furthermore, another mechanism by which CBD performs its anti-inflammatory action is mediated by interaction with the Transient Po- tential Vanilloid Receptor Type 1 (TRPV1). TRPV1 receptor is a nonselective cation channel that, when activated, allows the influx of Ca2+. The sensitivity but also the density of TRPV1 is increased during neuro-inflammatory conditions. The binding of CBD to TRPV1 leads to a desensitization of these receptors, with a consequent reduction in inflammation [20]. The CBD also carry out a potent antioxidant activity, modulating the expression of inducible nitric oxide synthase and nitrotyrosine as well as reducing production of reactive oxygen species [21]. CBD is also generating interest due to its therapeutic properties such as antidepressant [22], antipsychotic [23], analgesic [24], and antitumor [25]. In addition, it has been shown that CBD can significantly reduce two important forms of anxiety, namely obsessive-compulsive disorder [26] and post-traumatic stress disorder [27,28].

Moreover, for a long time, the CBD has been investigated for its anticonvulsant effects [29,30,31]. Several studies confirmed its efficacy in the treatment of epileptic seizures, espe- cially in pediatric age [32,33]. In 2016, the first results of phase III clinical trials showed bene- ficial effects of CBD (Epidiolex®; GW Pharmaceutical, Cambridge, UK) in treatment-resistant seizure disorders, including Lennox-Gastaut Syndrome (LGS) and Dravet syndromes (DS).

Epilepsy is a chronic neurological disorder. About 30% of epilepsy patients are affected by Treatment-Resistant Epilepsy (TRE) due to the failure of common anti-epileptic therapies [34]. This form of epilepsy is characterized by recurrent seizures that negatively affect the quality of life.

The purpose of this review is to provide an overview of recent clinical trials registered on Clin- icalTrial.gov. These trials study the use of different CBD formulation in patients affected by severe forms of drug-resistant epilepsy. Moreover, we have described studies approved by local ethics committees published in PubMed.

2. Epilepsy

According to the World Health Organization, epilepsy affects more than 50 million people worldwide. Epilepsy is the most common neurological disorders characterized by recurrent seizures [35]. A “seizure” is a paroxysmal transient phenomenon determined by an abnormal excessive or synchronous neuronal activity in the brain [36]. Epilepsy can also cause deficit sensorimotor, cognitive, compromising quality of life and an increased risk of premature death [37]. The International League Against Epilepsy, according to the point of onset, classifies epileptic seizures into focal, generalized and unknown seizures [38]. Focal convulsions caused by an anomalous electrical activity in a circumscribed part of the brain and are classified into simple and complex. Simple focal convulsions are characterized by motor, sensory and sensory manifestations without loss of consciousness. On the contrary, complex focal convulsions in- volve a loss of consciousness [39]. Generalized seizures begin in one or more areas of the brain and can then spread to the entire brain. Generalized seizures are divided into crises absences, characterized by a rapid and transient loss of consciousness; tonic crises that cause muscle stiffening; atonic crisis, characterized by loss of muscular control; clonic seizures that cause rhythmic muscle movements; myoclonic seizures, characterized by muscle contraction and localized tremors. Finally, tonic-clonic seizures represent the most serious type of epileptic seizures, last about 5–10 min and are characterized by intense generalized contractions to the whole body [39,40]. The unknown seizures are called so when the beginning of a seizure is not known. These seizures can also be defined as "epileptic spasms" characterized by sudden ex- tension or flexion of the limbs. Is defined Secondary Epilepsy when the onset is caused by sev- eral factors such as head trauma, infectious diseases (meningitis, AIDS, viral encephalitis), de- velopmental disorders, alcohol or drug abuse, and other pathological conditions (brain tumors, stroke).

The most well-known epilepsies are DS, Sturge-Weber Syndrome (SWS), Tuberous Sclerosis Complex (TSC) and West Syndrome (WS) and LGS. DS is a rare encephalopathy, which has its onset in the first year of life [41]. DS is associated with the mutation in the gene encoding the α1 subunit of the voltage gated sodium channel (SCN1A) [42]. SWS is caused by a somatic mutation of the GNAQ gene (9q21) that encodes the Gq protein, involved in the intracellular signal of several G protein-coupled receptors that control the function of various growth fac- tors and vasoactive peptides [43]. Patients manifest neurological abnormalities of variousde- grees, focal epileptic seizures [44]. TSC is an autosomal dominant disease, caused by a muta- tion of two genes: TSC1 (localized on chromosome 9p34.3) that encodes for hamartin and TSC2 (localized on chromosome 16p13.3) that encodes for tubulin. Often TSC patients present generalized epilepsy. WS or Infantile Spasm (IS) is the epileptic encephalopathy. This syn- drome is characterized by genetic heterogeneity and the mutated gene most frequently ob- served in patients with this syndrome is CDKL5 (cyclin-dependent kinase-like 5) [45]. WS is characterized by the association between axial spasm discharges and psychomotor retardation [46]. LGS is a severe epileptic encephalopathy of childhood. This syndrome is a rare condition likely associated with a genes mutation. Nevertheless, to date, it is quite unclear how the in- volved genes may cause this syndrome mainly characterized by recurrent seizures from early in life. An epileptic form that does not respond to therapy with at least two or three appropriately selected anti-epileptic drugs (AEDs) is defined as TRE and this is estimated to affect 30% of patients [47,48].

3. Common Antiepileptic Drugs AEDs are the mainstay for the treatment of epilepsy and are intended to mitigate seizures. Epileptogenic discharges occur as a result of neuronal hyperexcitability caused by voltage-de- pendent ion channels and neurotransmitter concentrations alteration. AEDs primarily act re- ducing neuronal excitability blocking excitatory neurotransmitter action such as glutamic acid and enhancing inhibitory neurotransmitters such as γ-aminobutyric acid (GABA). Further- more, the antiepileptic actions of most AEDs are due to the modulation of voltage-gated ion channels such as sodium (Na+) and calcium (Ca2+). The neuronal Na+ and Ca2+ channels are responsible for the rise of the action potential and for the intrinsic excitability control of the neuronal system [49]. Some AEDs act inactivating a single voltage-dependent channel while others instead simultaneously inactivate bothchannels. Both of these mechanisms result in a reduction for neuronal hyperexcitability. Examples of drugs that perform interacting with a single channel are phenytoin that selectively blocks the Na+ channel [50] and ethosuximide that blocks the T-type Ca2+ channel [51]. Instead, carbamazepine, lamotrigine, oxcarbazepine and zonisamide control seizures blocking both these voltage-dependent ion channels [52].

There are also anti-epileptics that act enhancing the GABAergic system. GABA is the main inhibitory neurotransmitter of the nervous system that acts on GABA receptors, ligand-depen- dent ionic receptors that increase chlorine conductance. AEDs are responsible for increasing GABA transmission reducing neuronal excitability. Drugs that exert their action through these mechanisms are benzodiazepines, phenobarbital, stiripentol, tiagabine and vigabatrin. Benzo- diazepines (such as clobazam diazepam, lorazepam, clonazepam) phenobarbital and stiripentol enhance the inhibitory transmission of GABA by allosteric activation of the GABAA receptor thus increasing the frequency of chloride (Cl–) channel openings [53,54]. Vigabatrin, instead, is an inhibitor of GABA transaminase, the enzyme responsible for the catabolism of GABA [55]. In addition to enhancing the inhibitory transmission of GABA, other drugs exert their antiepileptic action, also exploiting the blockage of the Ca2+ and Na+ channels. Among the AEDs that perform their action through these effects are included, felbamate, lamotrigine and topiramate [56]. Other drugs are valproic acid and levetiracetam that perform their mechanism of action enhancing the transmission of GABA and blocking Ca2+ channels [57,58].

The anticonvulsant and neuroprotective efficacy of some drugs is also given by the inhibitory action of neurotransmitters, such as glutamate. Glutamate (or glutamic acid) is the most com- mon excitatory neurotransmitter and is responsible for excitatory transmission on neurons. Fel- bamate and topiramate also perform their mechanism of action inhibiting glutamate thus de- creasing l’ hyperexcitability neuronal [59,60]. The choice of drugs is mainly linked to the identification of the type of seizure and epileptic syndrome. For patients with epilepsy, effective seizure control is the determining factor for a good quality of life. AED dosages must be individualized to maximize therapeutic effects and avoid side effects. The early childhood epilepsy syndrome such as DS, LGS and WS present no easy medical management due to the fact that subjects often show convulsion resistant to the available treatment. Therefore, of safe and effective therapies arenecessary to reduce the risk of neurological sequelae. The drugs preferentially used in particular forms of pediatric epilepsy are phenobarbital, phenytoin, benzodiazepine, topiramate, levetiracetam and valproic acid [61].

4. Cannabidiol and Molecular Targets in Epilepsy

CBD shows a low affinity for endocannabinoid receptors and it carries out its mechanisms of action by interacting with other molecular

targets. One of the most important ion channel tar- gets towards which the CBD shows a high affinity is the Transient Receptor Potential Vanilloid (TRPV).

Specifically, TRPV1 is a non-selective channel that shows a high Ca2+ permeability and is in- volved in the modulation of seizures and in epilepsy. In fact, when active, it promotes the re- lease of glutamate and the increase in Ca2+, with consequent neuronal excitability [62]. The antiepileptic action of CBD does not seem to be due to direct interaction with these molecular targets. However, it has been observed that the CBD agonist action towards TPRV1 determines one a desensitization of these channels with consequent normalization of intracellular Ca2+ [63]. T-Type Ca2+, are another class of ion channels with which CBD interacts. These chan- nels control Ca2+ peaks in neurons and they are involved in the regulation of cell excitability. The activation of these channels due to a hyperpolarization of the membranes of neurons deter- mines an increase in the concentration of intracellular Ca2+, in this way the T-Type Ca2+ chan- nels increase the excitability of neurons. This mechanism is often observed in pathophysiologi- cal conditions such as epilepsy [49]. The interaction of the CBD with the T-type Ca2+ channels causes a blockage of these channels, this mechanism could be responsible for the antiepileptic action, even if there are no studies available that confirm this. Receptors represent other molec- ular targets that have been evaluated to describe their potential role in epilepsy through interac- tion with CBD. Serotonin receptor (5-hydroxytryptamine [5-HT]) belonging to the superfamily of the G protein-coupled receptors are divided into seven distinct classes (5-HT1 to 5-HT7). These receptors may depolarize or hyperpolarize neurons, modifying the conductance and/or concentration ionic within the cells. This suggests that 5-HT receptors are involved in epilepsy even though their role is still not entirely clear [64]. CBD shows a high affinity towards two subtypes of serotonin receptors: 5-HT1A e 5-HT2A. These receptors can have different func- tions and regulatory characteristics, in fact, for example, the activation of 5HT1 receptors in the hippocampus causes an increase of neurotransmission; in contrast, in raphe nuclei, activation of 5-HT1A receptors produces the inhibition of serotonergic neurons [65]. The dysregulation of brain neurotransmission mediated by 5-HT2 might results responsible for the pathophysiology of depression and epilepsy [66]. However, although the role of serotonin receptors in epilepsy is unclear, 5-HT1A e 5-HT2A subtypes may represent a valid therapeutic target through which CBD can perform its anti-epileptic action [61,67]. Opioid receptors (OR) are G-protein-cou- pled receptors involved in a variety of brain disorders, including epilepsy [68,69]. The CBD at high micromolar concentrations determines the blocking of μ and δ OR, and this block would seem to generate anticonvulsant actions, even if there are still no studies to support this theory. The CBD also shows a good affinity towards the orphan G-protein-coupled receptor (GPR55), a class of receptors involved in the modulation of the synaptic transmission. The agonist action of CBD towards these receptors would seem to attenuate synaptic transmission with conse- quent antiepileptic effects [70].

An important enzyme target of CBD involved in epilepsy is cytochrome P450 (CYP450). CBD inhibits CYP450 [71], but this mechanism does not seem to be directly involved in the antiepileptic mechanism. It seems to be responsible for the hepatic metabolism of a variety of AEDs, as shown by the combined administration of CBD and clobazam (CLB) [72].

5. Cannabidiol: Clinical Trials for Epilepsy

In the last decades, some clinical studies were conducted in order to investigate the potential effects of the efficacy of CBD in the management of epilepsy. This review provides an over- view of the studies recorded on http://clinicaltrial.gov, distributing them into complete trials (see Table 1) and ongoing trials. All trials test the use of CBD as an adjunct to common AEDs and most of them assess the efficacy and safety of CBD especially in infants, children and teenagers. Trials exploring the combination of CBD/Δ9-THC have been excluded. In addition, we included further studies that have been approved by local ethics committees (see Table 2). In the description of these clinical trials, attention was also paid to the possible interactions of CBD with other anti-epileptic drugs.

Table 1

Completed cannabidiol clinical trials in epilepsy (https://clinicaltrials.gov/). The table shows the efficacy and safety of CBD in different forms of epilepsy. In all studies, CBD is used as adjunctive therapy to con- ventional antiepileptic drugs. Identifier

Study Title

A Study to Evaluate the Safety, Tolerability and Efficacy of Oral Administration of PTL101 (Cannabidiol) as an Adjunctive Treatment for Pediatric Intractable Epilepsy

Cannabidiol Oral Solution in Pediatric Participants With Treatment- resistant Seizure Disorders

Cannabidiol Oral Solution for Treatment of Refractory Infantile Spasms

Cannabidiol Oral Solution as an

Subjects

Conditions

Pediatric Intractable Epilepsy

CBD Dose

25–450 mg/kg/day

Concomitant AEDs

-

Efficacy

-

NCT02987114

16 children (2 to 15 years)

61 children (1 to 17 years)

9 infants (6 to 36 Months)

52 children

NCT02324673

NCT02551731
Resistant Seizure Disorders
Refractory Infantile Spasms
10, 20, 40 mg/kg/day
20–40 mg/kg/day
-
-
Improvement of illness
Complete resolution of spasm in 14.3% of children after 14 days of treatment

CBD: Cannabidiol; TEAEs: Treatment-emergent adverse events; SAEs: serious adverse events; AST: aspar- tate transferase; ALT: alanine transferase.

Table 2
Data obtained from trials authorized by local ethics committees (https://www.ncbi.nlm.nih.gov/pubmed/). The table shows the efficacy and safety of CBD in different forms of epilepsy. In all studies, CBD is used as adjunctive therapy to conventional antiepileptic drugs.

Study Design					
A prospective, open-label, expanded access study					
Subjects					
214 children and adults (1 to 30 years)					
Children and adults (1 to 30 years)					
55 children and					
Conditions					
Drug Resistant Epilepsy					
CBD Dose					
Initial 2–5 mg/kg/day titrated to maximum dose of 50 mg/kg/day					
Initial 2–5 mg/kg/day, titrated to maximum dose of 50 mg/kg/day					
Initial 5 mg/kg/day titrated to					
Concomitant AEDs					
CLB Valproate					
Efficacy					
The median reduction in monthly motor seizure was of 36.5%					
Overall quality of life significantly improved in 48 patients, The median monthly seizures frequency was 13.9					
Median monthly convulsive seizure frequency					
Safety Ref					
Treatment-related SAEs were recorded in 20 patients; SAEs were reported in 30% of					
patients. [83] Thrombocytopenia					
and elevated liver function test in patients with concomitant valproate					
- [86]					
A serious treatment- emergent AEs such as status					

CBD: Cannabidiol; TEAEs: Treatment-emergent adverse events; SAEs: serious adverse events.

5.1. Completed Clinical Trials

All clinical studies (phases 1, 2 and 3) reported below assess the safety and/or efficacy of CBD in addition to common AEDs. Most of these studies enrolled pediatric patients (0.5 to 17 years) with diagnoses of genetically based epilepsy, LGS, DS and WS, resistant to common antiepileptic treatments. The following trials collect data on administration short-term CBD (from 10 days to 3 months).

A phase 1/2 clinical trial NCT02324673 has evaluated efficacy and safety of the CBD oral so- lution at three different doses (10, 20 and 40 mg/kg/day) administered for 10 consecutive days in sixty-one children (1–17 years) with drug-resistant forms of epilepsy. It was to assess the plasma concentration of the CBD and its metabolite (7-hydroxycannabidiol, 7-OH-CBD) in blood samples collected at baseline, at day 1 and at day 10 post-treatment at different time points (1, 2, 4, 8, 12, 24, 48, 72 h). On day one, the plasma concentrations of CBD and its me- tabolite increase in a dose-dependent manner. These levels, instead, decrease at the 10th day. Moreover, both at the first and at the tenth day CBD concentrations are double those of its me- tabolite. A negative change in clinical global impression of severity score at the end of treat- ment has shown an improvement of illness in all dosage. An improvement, in a dose-dependent manner, has also been observed in daily seizure activity. Serious Adverse Events (SAEs) such as apnoea, skin eruption and thrombophlebitis, were observed in 5% of patients that received medium-dose CBD and in 9.5% that received high-dose. Further no-serious Adverse Events (AEs), such as anaemia, gastrointestinal disorders (diarrhoea, flatulence, constipation, gastroe- sophageal reflux disease, nausea), nervous system disorders (somnolence, psychomotor hyper- activity, seizure, ataxia) were respectively observed in 65% of patients treated with low-dose CBD, in 45% treated with medium dose and in 80.95% treated with high-dose. The results of this study showed that CBD can be considered safe and tolerable even at high concentrations.

A phase 2, multi-center clinical trial (NCT02551731) enrolled 9 children (6 months to 36 months) with a diagnosis of WS (Infantile Spasms) who have not responded to first-line thera- pies. CBD in oral solution was administered at the initial dose of 20 mg/kg/day or 40 mg/kg/day.

The protocol provided the division of the study into two parts: Part A and Part B. In Part A was evaluated the efficacy, defined as complete resolution of spasms and hypsarryth- mia (if present at baseline) confirmed by video-electroencephalogram (EEG) and safety at day 14 of treatment. Instead, in Part B was evaluated the efficacy and the long-term safety up to 64 weeks of treatment. In Part A, 14.3% of children showed complete resolution of spasms and hypsarrhythmia (when present at baseline) on day 14. Only in 33% showed AEs, while no SAEs were recorded. No results of Part B of the clinical trial were recorded because only one subject has completed the study. Then, these results confirm the efficacy and safety of the CBD oral solution after 14 days of treatment.

The patients that concluded the clinical trials NCT02324673 and NCT02551731 have been involved in the completed phase 3 open-label clinical-trial NCT02318602. The participants were divided for an age range into three groups: infants (1 to <2 years of age), children (2 to <12 years of age) and teenagers (12 to <17 years of age). All individuals continued the treat- ment with CBD at the same dose of trials NCT02324673 (10, 20 and 40 mg/kg daily) and NC- T02551731 (20 mg/kg daily) for 48 weeks. The first outcome of this clinical trial was to evalu- ate the safety of CBD as adjunctive therapy for children with treatment-resistant convulsive disorders. Patients following treatment with established AEDs were continued uninterrupted, dose adjustments were allowed if necessary based on safety concerns or changes in seizure control. SAEs such as seizures, status epilepticus and mental status changes occurred in 77.78% of infants, in 38.46% of children and 0% of teenagers. No serious AEs (anemia, diar- rhoea, constipation, vomiting, infection of the upper respiratory tract, nasopharyngitis, otitis media and influenza) occurred in 88.89% of infants, in 92.31% of children and 88.24% of teenagers. In all patients, no significant changes were observed as respect to baseline in labora- tory values or in vital signs. These results show that while the administration of CBD cannot be considered safe in infants, but it was generally well tolerated in adults.

The multicenter, open-label clinical trial, NCT03196934, is an extension of the NCT02318602 trial. The aim of this study is to assess the long-term safety of CBD oral solution as an adjunc- tive treatment for pediatric subjects with a treatment-resistant seizure. No result is available today.

Six randomized, double-blind, placebo-controlled studies were funded by GW Pharmaceuticals for evaluated the activity of the new formulation of purified CBD oral solution (GWP42003-P or Epidiolex), an epileptic medication and now Food and Drug Administration (FDA) ap- proved for the treatment of seizures associated with DS and LGS in patients two years of age or older.

The first clinical trial GWPCARE1 was divided into two parts: Part A (NCT02091206) and Part B (NCT02091375). NCT02091206, a double-blind randomization study (phase 2), to evaluate the safety of multiple doses of the CBD oral solution (GWP42003-P) in 34 children (4 to 10 years) with DS. All patients before enrolment had to have stabilized all AEDs at least 1 month before and the therapy stability had to be maintained during the study. Participants were randomized to one of the three doses (5, 10 and 20 mg/kg/day) of active drug or placebo at a 4:1 ratio. In addition, patients had to take their usual dose of antiepileptic drugs 2 h before CBD administration. The primary outcome was to assess the incidence of Treatment-Emergent AEs (TEAEs). A pharmacokinetic evaluation was also performed by measuring the plasma concentrations of CBD and its metabolites 7-OH-CBD and 7-carboxycannabidiol (7-COOH- CBD), and of the most common antiepileptic drugs taken by patients. Serious TEAEs, such as pyrexia and convulsions, occurred in five patients: one at the dose of 5 mg/kg/day, two at the dose of 10 mg/kg/day, one at the dose of 20 mg/kg/day and one in the placebo group. No seri- ous TEAEs (such as pyrexia, somnolence, decreased appetite, sedation, vomiting, ataxia and abnormal behaviour) reported in 80% of patients at 5 mg/kg/day of CBD, in 62.5% at the 10 mg/kg/day dose, in 77.78% at the 20 mg/kg/day dose and in 85.71% of placebo group. SAEs such as status epilepticus, convulsion, parvovirus infection, rash maculopapular, occurred in 10% of patients who received 5 mg/kg daily of CBD, in 25% at the 10 mg/kg daily, in 11.11% at the 20 mg/kg daily and in the 14.29% in placebo group. CBD was generally well-tolerated at the 5–20 mg/kg/day dose range. Elevated transaminases (ALT or AST) were only reported with concomitant use of valproate. The study showed that exposure to CBD and its metabolites increased in a dose-dependent manner, and 7-COOH-CBD was the most abundant circulating metabolite at all doses and times. In fact, at the end of treatment, 7-COOH-CBD levels were 13–17 times higher than those of CBD. The results also showed a pharmacokinetic interaction of CBD with CLB, resulting in an increment of the metabolite N-desmethylclobazam [N-CLB] in plasma exposure of the patients. An elevation in N-CLB, was absent in patients co-adminis- tered with stiripentol, possibly reflecting prior inhibition of the CYP2C19 isoenzyme [73].

All doses of CBD were well-tolerated and the 20 mg/kg/day dose was chosen by the for Part B (NCT02091375) study. NCT02091375 enrolled 120 children (2 to 10 years) with DS and drug- resistant epileptic seizures. Patients received either the CBD oral solution at a dose of 20 mg/kg/day (n = 61) or placebo (n = 59), for 14 weeks, in addition to the standard antiepileptic treatment. During CBD-treatment, SAEs (status epilepticus, convulsion and somnolence) oc- curred in 16.39% of patients and in 5.8% of the placebo group. Instead, non-serious AEs (diar-rhoea, vomiting, pyrexia, fatigue, upper respiratory tract infection, nasopharyngitis, decreased appetite, somnolence, lethargy, headache, convulsion, cough, irritability, gamma-glutamyl- transferase increased, transaminases increased, weight decreased) occurred in 75.41% of pa- tients who had taken CBD at the dose of 20 mg/kg/day and in 47.46% of the placebo group. The results suggested that, following the administration of CBD, the median frequency of seizures decreased from 12.4 to 5.9, compared to a decrease from 14.9 to 14.1 in the placebo- treated group. In 43% of patients treated with CBD and in 27% of patients in the placebo group occurred a reduction in seizure frequency by 50% or more and 3 patients were free of seizures [74]. Although the administration of CBD has caused high rates of AEs, CBD appears to be efficacy in the treatment of patients with DS.

Subsequently, GW launched a second Phase 3 trial, GWCARE2 (NCT02224703), to evaluate DS patients' responses to either a low (10 mg/kg/day) or a high dose (20 mg/kg/day) of GW- P42003-P for 14 weeks. The study, still recruiting, plans to enroll 150 participants, both chil-dren and adults (2 to 18 years). The results are not available.

The phase 3 clinical trial NCT02224560 (GWPCARE3) included 225 patients with LGS (2 to 55 years) with two or more seizures/week. This study was to evaluate the safety and efficacy of the CBD oral solution (GWP42003-P) as an adjunctive treatment of other antiepileptic drugs. The patients were divided into 3 groups and treated with CBD at the dose of 10 mg/kg/day or 20 mg/kg/day or with placebo for 14 weeks. During the treatment, SAEs such as pneumonia, status epilepticus, elevated aspartate aminotransferase concentration, elevated

alanine amino- transferase concentration and elevated γ-glutamyltransferase concentration, occurred in 19.40% of patients treated with 10 mg/kg/day of CBD, in 15.85% treated with 20 mg/kg/day and in 10.53% of the placebo group. Increases in serum aminotransferase concentrations occurred only in patients treated with placebo. While, non-serious AEs (diarrhea, vomiting, decreased appetite, pyrexia, fatigue, somnolence, upper respiratory tract infection, nasopharyngitis) have been observed in 53.73% of 10 mg/kg daily group, in 76.83% of 20 mg/kg/day group, and in 52.63% of the placebo group. A median percent reduction from baseline in drop-seizure fre- quency was 37.2% in the 10 mg/kg/day CBD group, 41.9% in the 20 mg/kg/day CBD group, and 17.2% in the placebo group. The results reported by the authors show that the addition of the CBD to conventional antiepileptic therapy reduces the frequency of seizures in a dose-de- pendent manner [75].

The phase 3 clinical trial NCT02224690 (GWPCARE4) included 171 patients (aged between 2 and 55 years) with a diagnosis of LGS. Participants had to have taken one or more antiepileptic drugs (the most used was lamotrigine, valproate and CLB) at a stable dose for at least 4 weeks prior to screening as well as interventions for epilepsy. The first endpoint was to aim the effica- cy of the CBD oral solution (GWP42003-P) as adjunctive treatment in reducing the number of drop seizures when compared to the placebo. The secondary endpoint was to assess the safety of CBD by measuring AEs using standard severity measures. Individuals were divided into two groups: 85 received placebo and 86 received a CBD at a dose of 20 mg/kg/day for 14 weeks. SAEs (pneumonia, viral infection, alanine aminotransferase increased, aspartate amino- transferase increased, γ-glutamyltransferase increased) occurred in the 23.26% of CBD group and in 4.71% of patients in the placebo group. Serious TEAEs (increased levels of alanine aminotransferase, aspartate aminotransferase and γ-glutamyltransferase) occurred in four pa- tients in the CBD group. Instead, the most common no serious-AEs (vomiting, diarrhoea, loss of appetite and drowsiness) occurred in the 61.63% of CBD group and in 50.59% of patients in the placebo group. After 14 weeks of treatment, the monthly frequency of seizures decreased by a median of 43·9% from baseline in the CBD group. A reduction in seizures frequency of 50% or more, was reported in 44% of patients in the CBD group and in 24% of patients in the placebo group. The study found that in many patients treated with antiepileptic drugs that in- cluded CLB, a higher onset of somnolence was observed. High levels of transaminases were recorded in patients treated with valproate. Nevertheless, the high rate of AEs, the results showed that the administration of long-term CBD oral solution in patients with LGS deter- mines the reduction in seizure frequency compared to placebo [76].

Subsequently, all patients who completed the treatment period in NCT02091206, NC- T02091375, NCT02224703, NCT02224560 or NCT02224690 were included in the sixth clini- cal trial GWPCARE5 (NCT02224573). The results of this study will help to understand the safety of CBD administered over long periods. Patients received an oral solution of CBD (100 mg/mL), titrated from 2.5 to 20 mg/kg/day over a 2-week period, in addiction with their exist- ing treatment. The median treatment duration was 274 days. SAEs such as status epilepticus and convulsion occurred in 29.2% of patients. Commonly reported AEs (diarrhoea, pyrexia, decreased appetite and somnolence) occurred in 93.2% of patients. 17.2% of patients from GWPCARE1 that taking valproic acid, had liver transaminase elevations. In patients from GWPCARE1 Part B, the monthly frequency of seizures from baseline decreased by a median of ranged from 38% to 44% in 12-week periods up to week 48.85% of patients reported an im- provement in the overall condition after 48 weeks of treatment. This trial showed that long-term CBD treatment was safe and efficacy to reduce seizure frequency in patients with treat- ment-resistant DS [77].

A randomized controlled trial NCT02565108 (phase 2) included twenty patients (aged 18 to 65 years) with diagnosed epilepsy treated with CLB. This study examined the possible drug-drug interactions between CLB and CBD. Participants before enrolment followed a stable therapy for at least a month with antiepileptic drugs, including CLB. Patients received CBD oral solu- tion (GWP42003-P) at a dose of 20 mg/kg/day after taking CLB for 21 consecutive days. 75% of patients in the CBD group and 50% of patients in the placebo group showed non-serious AEs (diarrhea, nausea, vomiting, dizziness, somnolence, sedation, dermatitis). Results showed that all participants reduced the maintenance dose of CBD of 10%/day. Eighteen participants who completed trial NCT02565108 were transferred to the open-label extension (OLE) trial NCT02564952. The OLE phase was a safety study. Initially, all partici- pants received CBD at a dose of 20 mg/kg/day, thereafter the dose was decreased or increased to a maximum of 30 mg/kg/day. All individuals during the study continued to receive CLB. In addition to CLB, participants could not take more than two other AEDs during the study. Only seven of the 18 participants completed the study, 11.11% showed SAEs (status epilepticus, seizure, alanine aminotransferase abnormal, aspartate aminotransferase abnormal γ-glutamyl- transferase abnormal). While, 94.44% of patients presented no-serious AEs such as diarrhoea, vomiting, headache, hyponatraemia, dizziness, seizure, somnolence, irritability, respiratory tract infection. The high rate of AEs in the concomitant use of the CBD and CLB for pro- longed periods of time may be unsafe.

The clinical trials of phase I NCT02695537 and NCT02700412, will evaluate prospectively and longitudinally the safety and tolerability of CBD oral solution (Epidiolex) at various doses, between 5 mg/kg/day and 25 mg/kg/day with additional titration in some cases up to 50 mg/kg/day. These two trials will enroll both 100 patients with drug-resistant epilepsy. Clinical trial NCT02695537 will enroll patients aged 1 to 18 years, while the NCT02700412 patients aged 17 to 99 years. However, Gaston, et al. [78] evaluated possible CBD interactions with antiepileptic drugs typically used in 39 adults and 42 children of these trials. An analysis was carried out to check for non-uniform changes in both the CBD dose and the dose of other AEDs. In the two combined arms (pediatric and adult) the results recorded linear increases in serum levels of topiramate, rufinamide and [N-CLB] and linear decreases in CLB levels corre- late with increasing CBD does. However, there were no significant changes in the levels of oth- er AEDs analyzed (valproate, levetiracetam, phenobarbital, clonazepam, phenytoin, carbamazepine, lamotrigine, oxcarbazepine, ethosuximide, vigabatrin, ezogabine, pregabalin, per- ampanel and lacosamide). During the study, six adults and eight children showed sedation. The intake of concomitant CBD and valproate resulted in high levels of AST and ALT. Liver function tests showed elevated damage greater than three times the normal limit in four children who dropped out of the study, while the damages of about twice the upper normal limit in eight adults were resolved with valproate withdrawal. A major onset of somnolence following the concomitant administration of CBD and CLB and high levels of transaminases following co-administration of CBD and valproate was also recorded in another study [73] and in clinical trial NCT02224690. In conclusion, the results obtained by the researchers show that the use of CBD with other drugs can be considered safe. On the contrary concomitant use of CBD with valproate is not recommended as a

significant liver dysfunction has been observed. Probably because CBD enhances the toxic action of valproate. The interaction between CBD and CLB was also highlighted. Since both of these drugs are metabolized of the cytochrome P450 path- way, this interaction can often induce high plasma levels of n-CLB. Therefore, it is important to monitor this drug-drug interaction. However, as the adverse effects occurring, in this case, are not serious, the concomitant use of CBD with CLB can be considered safe and above all effective, especially in pediatric patients with refractory epilepsy. Part of the results of NC- T02695537 and NCT02700412 were described by Szaflarski, et al. [79]. The study showed the efficacy and safety of Epidiolex in 72 children and 60 adults. The results obtained show an av- erage reduction of all types of seizures of 63.6% with difference significant between baseline and 12 weeks. The reduction in seizures seems to have remained stable, in fact, there were no significant differences between 12 and 24 weeks and between 24 and 48 weeks. The severity of the seizures assessed by the Chalfont Seizure Severity Scale (CSSS) also showed an improve- ment from a baseline score of 80.7 to enroll at 39.3 at 12 weeks with CSSS scores stable even between 12 and 24 weeks and between 24 and 48 weeks. The analysis of AE Profile indicates a significant improvement in the presence/severity of adverse events between the baseline and 12 weeks with stable AEDs thereafter without significant differences between 12 and 24 weeks and between 24 and 48 weeks. The results of this study show significant improvements in the profile of adverse events, in the severity of crises and in reducing the frequency of seizures as early as 12 weeks; improvements that have been maintained during the 48 weeks of treatment.

A clinical trial of phase II NCT02987114, is an open-label, single-center trial, that recruited 16 children (aged 2-15 years), with intractable epilepsy. The aim of this trial was to evaluate the safety, tolerability and efficacy of oral administration of PTL101 (formulation of seamless gelatin matrix green beads containing CBD) as adjunctive therapy for pediatric intractable epilepsy. Patients at least 4 weeks before enrolment had to have stabilized the doses of antiepileptic drugs. This clinical trial has included 4 weeks of observation of clinical parame- ters and 13 weeks of CBD treatment at an initial dose of 25 mg/kg daily up to the maximum dose of 450 mg/kg. Subsequently the patients were monitored for 2 weeks. The results of this study, not yet available.

The results obtained from all the completed studies show that CBD is a safe compound when combined with common AEDs. An aspect of particular interest concerns the association of CBD with valproate and CLB. In particular, some studies have shown that the association be- tween CBD and valproate leads to a reduction in liver function related to an increase in transaminases. This alteration has been shown to be reversible and not to cause permanent liver damage. Pharmacokinetic studies have shown that CBD determines, as associated with CLB, a plasma increase in the metabolites of this benzodiazepine. All trials reporting efficacy data show that CBD is able to reduce the frequency of seizures.

5.2. Ongoing Clinical Trials

In the last 4 years, various tests have been started to evaluate the efficacy and safety of CBD as an antiepileptic. In this subsection, we have collected the trials that to date have not yet avail- able results as they are still in the patient recruitment phase.

Two phase 2 clinical trials NCT03355300 and NCT03336242 are expecting to recruit about 30 pediatric patients (3 to 17 years), with treatment-resistant childhood absence seizure. Both studies will include three experimental treatment cohorts (20, 30 and 40 mg/kg/day). NC- T03336242 will assess efficacy, safety, tolerability and pharmacokinetics of CBD oral solution after 4 weeks of treatment. This study will include a 4-week screening period and a 5 or 10 day titration period (depending on study cohort), a 4-week treatment period followed by 5-day ta- pering for doses >20 mg/kg/day and a 4-week follow-up period. Instead, NCT03355300 will evaluate the long-term (up to approximately 54 weeks) safety and tolerability of the CBD oral solution, monitoring the incidence of SAEs and AEs during and after treatment. For both tri- als, the final data collection and the results are expected by the end of the year 2019.

In the clinical trial NCT03676049, CBD will be administered as an adjunct to all current AEDs in 5–10 patients (aged between 5 and 19) with refractory epilepsy. The CBD oral solu- tion used for treatment, with prior approval from the National Institute on Drug Abuse was prepared at the University of Mississippi, and subsequently received FDA approval for com- passionate use. A dosing titration period will start with 100 mg/day, and will be titrated month- ly as tolerated based on clinical response, up to 300 mg/day. During the treatment period the patients will be subjected to control visits at the baseline, at the fourth, at the eighth and at the twelfth weeks. During these visits the efficacy of the treatment will be evaluated, observing the laboratory tests, quality of life of the patient, the profile of the side effects and the crisis count. Patients who after 3 months of treatment show stability could continue the use of CBD for an- other 3 months.

The clinical trial NCT02461706 will assess the safety and efficacy of CBD when administered as adjunctive therapy in 50 children (2 to 16 years) who have resistant to AEDs. Patients treat- ed with AEDs were to have stabilized doses at least 4 weeks prior to enrolling. The study es- tablished the starting dose of 25 mg/kg/day. Maximum dose titration should be achieved in most patients within 5 weeks. The patients will be clinically evaluated at baseline, once a month for three months and once every three months thereafter. In addition, to ensure the safe- ty of the study, all patients who reached the maximum dose (more than 600 mg of daily) of the CBD will be monitored at least once a month until the steady state of the maintenance dose was reached. A double-blind, randomized, placebo-controlled phase 3 trial (NCT02783092), is intended to evaluate the efficacy of the adjuvant use of CBD oral solution (200 mg/ml dissolved in corn oil), in patients with epilepsy. The estimated 126 patients (2 to 18 years) will be treated with CBD at the initial dose of 5 mg/kg/day, up to a maximum dose of 25 mg/kg/day. The primary outcome is to evaluate whether CBD treatment resulted in a 50% reduction in seizure frequen- cy compared to treatment with antiepileptic drugs after 30 days. The results of this study (esti- mated final data collection in August 2020) will make it possible to clarify the efficacy of CBD at different doses.

The phase I clinical trial NCT02286986 is a multi-center study that to investigate the pharma- cokinetics and dose-ranging tolerability, efficacy and safety of CBD (GWP42003-P), in 25 children and young adults (2 to 25 years) with epilepsy. The study was divided into two parts: Part A and Part B. Part A was used to evaluate the safety and tolerability of more ascending doses of GWP42003-P compared to placebo. The best-tolerated dose in Part A of the study was used to treat patients in Part B for 60 consecutive days. The antiepileptic efficacy of GW- P42003-P compared to placebo was evaluated by monitoring the incidence in convulsions, de- termining the plasma

concentration of GWP42003-P and its main metabolite following the escalation of multiple doses of GWP42003-P. Furthermore, was investigated the effect of GW- P42003-P on the pharmacokinetics of concomitant and cognitive function, sleep quality and daytime sleepiness were also observed. The trial is still active and the results have not been published, yet.

Two phases II clinical trials NCT02607904 and NCT02607891 want to verify the possible drug-drug interactions between GWP42003-P and two antiepileptic drugs, stiripentol or val- proate in patients with epilepsy. Both trials will enroll patients between 16 to 55 years. In the trials NCT02607904, patients will be treated up to a maximum dose of 30 mg/kg/day for 12 months. Instead, in NCT02607891 trials, the participants will be randomized into a 4: 1 ratio to receive GWP42003-P or corresponding placebo. The hypothesis is that levels of stiripentol or valproate may be altered as a result of using GWP42003-P. During treatment, CBD will be administered at a maximum dose of 20 mg/kg/day for 25 days. Participants had to take stiripentol or valproate and no more than two other AEDs during the blinded period of the study.

Two phases III clinical trials NCT02953548 and NCT02954887 intended to evaluate the effica- cy and safety of CBD oral solution (GWP42003-P; GW Pharmaceutical) in infants with WS (Infantile Spasms). These studies weredivided into 3 phases: a pilot safety phase; a randomized central controlled phase and an open-label extension phase. The NCT02953548 will be de- scribed in the pilot phase. Two cohorts of five participants will be enrolled sequentially. GW-P42003-P will be administered up to a maximum dose of 40 mg/kg/day for the 2-week treat- ment period. Instead, clinical trial NCT02954887 will be an extension trial that will recruit 202 infants (from 1 month to 24 months), for 1 year of treatment. The results expected from this study will allow observing if the administration of CBD will be effective in infants with WS.

Two phases III clinical trial NCT02544763 and NCT02544750 (GWPCARE6) will evaluate respectively, in a double-blinded phase and in an open-label extension phase, the efficacy of the CBD oral solution (GWP42003-P) as adjunctive therapy by monitoring the frequency of seizures in patients with TSC. NCT02544763 is expecting to recruit about 210 patients (1 to 65 years). All AEDs or interventions will be stabilized at least 1 month before the screening and the stability of the therapy will be maintained during the study. Patients will be treated with CBD at the dose of 25 or 50 mg/kg/day for 16 weeks. The efficacy of the CBD will be tested by evaluating the change in seizure frequency. Patients that will complete this blinded phase will be included in the NCT02544750 trial. The safety of CBD administration will be mea- sured based on the incidence of AEs. All participants will be dosed up to a maximum of 50 mg/kg/day. From these two trials the results, not yet available, will help to understand if the administration of CBD can lead to a decrease in the crisis in patients with TSC.

A phase 1/2 clinical trial NCT03014440, aim to determine the safety and tolerability of CBD (Epidiolex) in addition to the anti-epileptic treatments in use, in patients aged 1 to 20 years with drug-resistant epilepsy. Antiepileptic therapy followed by patients had to be stable for at least 1 month. To date, there is no information available regarding the treatment, the doses used and the results.

The NCT02660255 is an observational, open-label, flexible dose study. The aim of this trial is to evaluate the safety and efficacy of Epidiolex, in addition to common AEDs. The study will be recruited subjects aged 1-60 years with treatment-resistant epilepsy. Patients prior to enrol- ment will be treated with 1–4 AEDs on stable settings from least 1 month. Epidiolex will be administered for 1 year and 9 months. To date, no superior information and results are avail- able, yet.

The clinical trial NCT02397863 is an open-label, multi-center study including patients (1 to 18 years of age) with drug-resistant epilepsy. Patients are treated with CBD (Epidolex), the daily dosage is up to 25 mg/kg/day with optional up-titration to a maximal daily dosage up to 50 m/kg/day until the end of treatment. Treatment was provided for a total of 52 weeks. For this study the results have not been published, yet.

Clinical trial NCT02332655 (phase 1/2) aims to assess the tolerability and optimal dose of CBD to be used as a treatment in children and young adults with SWS and drug-resistant epilepsy to define the optimal dose of Epidiolex. The study involving the recruitment of the 10 patients (aged 1 months to 45 years) already in treatment with antiepileptic drugs. Patients treated with 1–5 basic antiepileptic drugs had to have reached stable doses for a minimum of 4 weeks prior to enrolment. Treatment will start with 2 mg/kg/day. The dose will be increased by 3 mg/kg/day after seven days and then by 5 mg/kg/day every seven days up to a maximum dose of 25 mg/kg/day given for 48 weeks. From the expected results potential efficacy of CBD in refractory crises in patients with SWS will emerge.

An open-label observational study NCT02556008 will evaluate the efficacy of pure CBD for the treatment of 25 children (1 to 17 years) with severe refractory epilepsy. The pure CBD used during treatment is not approved by the FDA, therefore, investigators conducted this study through the FDA's expanded access mechanism for compassionate use. CBD will be ad- ministered as an adjunct to all current anti-epileptic therapies. Patients had to undergo thera- peutic treatment with 1-3 basic antiepileptic drugs at stable doses for a minimum of 4 weeks prior to enrolment. The expected dosage of the study was 2 mg/kg/day for a first week, 3 mg/kg/day for the second week, 5 mg/kg/day for the third week up to a maximum dose of 25 mg/Kg/day. Seizure frequency will be assessed four weeks before the initiation of CBD, the next month, and at least every 3 months thereafter. The results of efficacy of CBD are not yet available. Data from these studies will be available soon, as the final data collection for many studies is expected by the end of 2019.

5.3. Clinical Trials Approved by Local Ethics Committees

In this subsection, clinical studies published in indexed journals have been described. Many of these manuscripts report the results obtained on long periods of treatment with CBD and pro- vide important efficacy data. In addition, the common antiepileptic drugs taken by patients in association with CBD are detailed.

The study conducted by Geffrey, et al. [72], approved by Massachusetts General Hospital (MGH) Institutional Review Board (IRB) evaluated the CBD interaction with CLB. The aim of this study was to evaluate possible interactions between CBD and CLB, assessing its efficacy, safety and pharmacokinetics. For this study, 13 patients (4 to 19 years) with refractory epilepsy and treated with CLB were recruited. Patients started taking CBD at a dose of 5 mg/kg/day and treated up by 5 mg/kg/day each week to a dose of 25 mg/kg/day, for 8 weeks. CLB was admin- istered daily at a stable dose of 0.5 mg/kg that was decreased during the study when side ef- fects were observed. The plasma levels of CBD, CLB and [N-CLB] were measured at baseline and at weeks 4 and 8 of treatment. The results of the efficacy study showed a 50% convulsion reduction in nine out of 13 subjects, corresponding to a 70% response rate. In two patients, however, there was an increase in the frequency of seizures during the treatment period, there- fore the dose of CLB was reduced. Increases in plasma levels of CBD, CLB

and its metabolite were recorded. Already in the fourth week, the mean of CBL levels had been an increase of 60 ± 80%, while the mean in [N-CLB] was an increase of 500 ± 300%. The results of the safety study show that in 77% subjects AEs were reported as somnolence (n = 6), ataxia (n = 2), irri- tability (n = 2), restless sleep (n = 1), urinary retention (n = 1), tremor (n = 1) and loss of ap- petite (n = 1). After adjusting the doses of CLB all AEs were resolved. Therefore, the results reported by the authors show an interaction between CBD and CLB, and that CBD influences [N-CLB] levels much more than CLB levels.

The efficacy of the interaction between CBD and CLB was also seen in a study conducted by Porcari, et al. [80]. This study was approved by the Vanderbilt University Institutional Review Board. In this retrospective study, the aim is to define the efficacy of CBD alone or in associa- tion with CLB in 209 children (≤ 19 years) with epilepsy. The duration of treatment in patients receiving CBD was 1.1 years, while patients received CBD + CLB for 1.3 years, and for 2.5 years received CLB. The reduction of antiepileptic drugs was seen in 21% of the CBD group, in 26% CBD + CLB and in 18% of CLB group. No-seizures were observed in 14% of patients in the CBD group, in 9% of patients in the CBD + CLB group and in 11% of patients in the CLB group. The results reported by the authors show a reduction of the crises > of 50% in 33% of the CBD group, in 44% of the CBD + CLB group and in 38% of the CLB group. It was also observed that LGS was the most commonly observed syndrome in all cohorts, and in these patients, the response rate was 58% with CBD, 52% with CBD and CLB and 40% with CLB alone. The sedation was the most common AEs reported in 36% of patients in the CLB group, in 7% of patients in the CBD + CLB group and in 0% of CBD group. This retrospective study suggests that CBD is useful in the treatment of refractory epilepsy with benefits that can- not be attributed to the interaction with CLB and increased levels of its active metabolite.

Morrison, et al. [81] conducted a pharmacokinetic study that evaluated the possible drug-drug interactions between CLB, stiripentol or valproate and CBD (Epidiolex). This study was ap- proved by the Independent Ethics Committee of the Foundation Evaluation of Ethics in Bio- medical Research, Assen, The Netherlands. For this open-label phase I study, were enrolled 78 healthy subjects. The primary outcome was to evaluate the interaction of multiple CBD admin- istration as a perpetrator drug with antiepileptic drugs (victim) at steady-state plasma concen- trations: CLB (and its active metabolite, N-desmethylclobazam); stiripentol and valproate (and its potentially hepatotoxic metabolite, 4-ene-VPA). On the contrary, was also evaluated the interaction of CBD (victim drug) and its metabolites 7-OH-CBD and 7-COOH-CBD at steady-state plasmatic concentrations, with multiple doses of CLB, stiripentol or valproate as perpe- trators drugs. CBD was given at 750 mg twice daily, CLB at 10 mg/kg/day, stiripentol at 750 mg and valproate at 750 mg twice a day. The results showed a significant interaction between CBD and CLB. When CLB was used as the victim drug, significant increases in its metabolite [N-CLB] were recorded. These increases are related to an inhibition of the CPY2C19. In addi- tion, the concentrations of the active metabolite 7-OH-CBD increased when was co-adminis- tered with CLB. Stiripentol, however, increased by 28% when it is at steady-state plasma con- centrations alone, and by 50% following co-administration with CBD. The 50% increase in stiripentol concentration may be caused by an inhibition of CPY2C19 by the CBD. Instead, co- administration of stiripentol with CBD not caused an increase in CBD concentrations, but caused a 29% increase of 7-OH-CBD and 13% of 7-COOH-CBD. The interaction of the CBD and valproate did not affect the pharmacokinetics of the two drugs. Regarding the safety study, six subjects were withdrawn due to adverse events; three when CLB was added to the steady- state CBD and three when the valproate was added to the steady-state CBD. Two subjects re- ported SAEs when CLB was co-administered to CBD. Moderate AEs were reported in eight subjects; instead mild AEs were reported in most subjects. The results obtained by the authors can be concluded by saying that the co-administration of drugs was moderately tolerated. Fur- thermore, the drug-drug bidirectional interaction noted when CLB was co-administered with CBD, suggests a dose reduction for CLB when administered with CBD.

Another study to evaluate the efficacy of the CBD oral solution (GWP42003-P) as a therapy for drug-resistant epilepsy in TSC was conducted by Hess, et al. [82] (approved by Mass- achusetts General Hospital Institutional Review Board and U.S. Food and Drug Administra- tion). Of the 56 patients enrolled in this study, only 18 patients (aged 2 to 31 years) were evalu- ated because they were affected by TSC. At the time of enrolment, patients were taking be- tween one and seven anti-epileptic drugs, such as lacosamide (n = 14), CLB (n = 10), leve- tiracetam (n = 7), lamotrigine (n = 5), valproic acid (n = 3) and rufinamide (n = 3). Treatment started at a dose of 5 mg/kg/day. This dose was increased by 5 mg/kg/day every week up to the initial maximum dose of 50 mg/kg daily, for 12 months. After the third month of treatment, doses of the CBD and concomitant AEDs could be adjusted monthly in almost all patients in order to optimize seizure control. 15 patients achieved the initial maximum dose of 25 mg/kg/day of CBD, while five achieved the highest dose of 50 mg/kg/day of the CBD, and at this dose, none reported CBD-related AEs. Instead, six patients decreased the dose of CBD during the study in order to alleviate AEs and interactions with concurrent AEDs. 66.7% of patients reported AEs and among them, drowsiness, ataxia and diarrhoea. Three months after the treatment, in four patients a reduction in seizure rate greater than 80% was recorded and one patient became seizure-free and he remained free until the twelfth month. The results also show that in patients took CBD and CLB the response rate after 3 months of treatment was 58.3% against 33.3% in patients who did not take CLB. Given the results reported by the au- thors in this study, the CBD can be considered valid and safe in the treatment of refractory epilepsy in the TSC.

Five patients enrolled in this study were included in another multicentre analysis of CBD ex- panded-access conducted by Devinsky, et al. [83] (in 11 epilepsy centers in the USA). The aim of this study was to assess safe, tolerated and effective of CBD (Epidiolex) in children and young adults with severe, intractable, treatment-resistant epilepsy (the most common epilepsy syndrome treated were DS and LGS). This study was approved by the institutional review boards at each study site. CBD was used in addition to anti-epileptic treatment. For this trial, 214 patients were enrolled (1 to 30 years); of these 162 patients after the first dose of CBD were monitored for 12 weeks and were included in the safety and tolerability analysis while 137 patients (64%) were included in the efficacy analysis. Patients started the treatment with CBD at the initial dose of 2–5 mg/kg daily up to a maximum dose of 50 mg/kg daily for 12 weeks. In the safety study, SAEs were observed in 30% of patients. Treatment-related serious AEs, such as status epilepticus, diarrhoea, pneumonia and weight loss were recorded in 20 in- dividuals. Instead, in 79% of patients showed no serious AEs, the most common were de- creased appetite, fatigue, somnolence, diarrhoea, convulsions, status epilepticus, sedation, lethargy. After 12 weeks of treatment, results showed a median reduction in monthly motor seizures of 36.5%. In the patients with DS (n = 32), the treatment led to a median reduction of monthly motor convulsions of 49%, in 16

patients a reduction of 50%. Instead, for patients with LGS (n = 30), an average reduction of 36.8% in motor crises was recorded. Findings ob- tained from this study showed that CBD seems to reduce the frequency of seizures and also shows an appropriate safety profile, even in patients with DS and LGS.

Another study conducted by Sands, et al. [84] assessed the long-term safety, tolerability and efficacy of CBD to children with refractory epilepsy. This study was approved by the Human Research Ethics Committee of the UCSF Benioff Children's Hospital. The CBD oral solution (Epidiolex) was administered in addition to other anti-epileptic treatments in 26 patients (aged 1 to 17 years). The doses of concomitant antiepileptic drugs had to be stable during the 4-week of baseline period and had to remain stable during the first three months of treatment. CBD was administered at the starting dose of 5 mg/kg daily and subsequently, weekly dosage was measured in increments of 5 mg/kg daily up to a maximum dose of 25 mg/kg daily. The dura- tion of therapy ranged from 4 to 53 months. The patients underwent blood tests performed dur- ing the baseline period, after 1, 2 and 3 months and thereafter every 3 months from treatment. Furthermore, the minimum concentrations of antiepileptic drugs were evaluated. The frequen- cy of seizures and AEs was monitored during the treatment period. The primary outcome of the study was to test the efficacy of CBD in terms of > 50% reduction in the frequency of motor seizures. Fifteen of 26 patients discontinued treatment, one due to a status epilepticus, one for severe weight loss, all others for lack of efficacy. Instead, six patients showed SAEs as sta- tus epilepticus (n = 3), catatonia (n = 2) and hypoalbuminemia (n = 1). 21 out of 26 patients reported no serious AEs among which the most frequent were: reduced appetite (n = 10), diar- rhoea (n = 9), and weight loss (n = 8). In patients showing significant weight loss, the doses of CBD were reduced. Changes in the concentrations of antiepileptic drugs were observed in four patients. Three of them reported increased CLB concentrations, one reported an increment in phenobarbital contractions. In three patients was observed an increment in aspartate amino- transferase and alanine transferase levels when CBD was co-administered with valproate. The reduction in the frequency of seizures > 50%, was rediscovered in 38.4% of patients after 3 months of treatment, in 56.7% after 6 months, in 42.3% after 9 months, in 38.4% after 12 months, 42.3% after 18 months and 34.6% after 24 months. In conclusion, after 24 months of treatment, of the 26 patients enrolled, only nine continued CBD as adjunctive therapy. Of these patients, seven had a 50% reduction in the frequency of motor crises, three of which remained completely free of seizures. Only seven of the nine patients who continued treatment showed a reduction in seizure frequency > 50% after 36 months. The results reported by the authors showed that long-term CBD results in a clinically significant reduction in seizure frequency, and a low percentage of SAEs. Moreover, because treatment was stopped after a few months in most patients, the number of patients exposed to CBD for a long time is low and the rate of adverse effects over time may be underestimated.

Using the same CBD formulation (Epidiolex) and the same administration doses Kaplan, et al. [85] conducted a study that was approved by the Federal Drug Administration for the use of Epidiolex in the treatment of pediatric medically refractory epilepsy in SWS. In this study, five patients (aged 1 months to 45 years) were enrolled. The maximum dose of 25 mg/kg daily was tolerated only by two patients, while in the other three the maximum tolerated dose was 20 mg/kg per day. Three participants withdrew from the study, two due to lack of efficacy (week 38 and week 9), while one due to the temporary increase in seizures during dose titration, but later re-enrolled. Three subjects remain in the extension phase of the study continued to take CBD for more than a year. All subjects reported at least one CBD-related adverse event during the study such as temporarily increased seizures, behavioral issues, increased aspartate amino- transferase and fatigue. All transient AEs resolved spontaneously after dose changes in con- comitant anticonvulsants or CBD. Seizure reduction above 50% was seen in two patients at weeks 14 and in three patients with bilateral brain involvement. Instead, subjects reported im- provements in quality of life during the treatment. As suggested by the results obtained from this study, CBD appears to be well tolerated and a valid candidate as adjunctive therapy for seizures management in individuals with SWS. Rosenberg, et al. [86] belonging to the same research group of Devinsky, et al. [83] prolonged the study for evaluating the Quality of Life of Childhood Epilepsy (QOLCE) before and after treatment with CBD (Epidolex). The study was approved by the NYU Langone Medical Center institutional ethics board. For this study were enrolled patients (aged 1–30) with intractable treatment-resistant epilepsy. In addition to the baseline antiepileptic drugs, patients were given CBD at the initial dose of 2–5 mg/Kg daily up to the maximum dose of 50 mg/Kg daily for 12 weeks. After 12 weeks of treatment with CBD, the median monthly seizures frequency was 13.9 and the median percent change from baseline was –39.4%. In addition, the results indicat- ed an improvement of 8.2 ± 9.9 points in patient QOLCE. In fact, patients showed an improve- ment in behaviour, in memory, in energy/fatigue, in control/impotence, in other cognitive func- tions and in global quality of life.

The same research group of Devinsky, et al. [87], conducted a study for evaluating the safety and efficacy of long-term CBD administration in patients with severe childhood-onset epilepsy, and with CDKL5 deficiency disorder and Aicardi, Doose syndromes and Dup15q syndromes. This study was approved by the IRB at each institution. For this study, 55 patients aged be- tween 1 and 30 were enrolled (with 55 in the safety group and 50 in the efficacy group). Pa- tients were given a pharmaceutical compound of Highly purified CBD (Epidolex). Treatment included 144 weeks of Epidiolex administration in addition to anti-epileptic therapies at the starting dose of 5 mg/Kg per day. During treatment, an increase of 2–10 mg/kg per day was carried out every two weeks up to the maximum dose of 50 mg/kg per day. The efficacy study showed that the percent change in median monthly convulsive seizure frequency for all pa- tients after treatment decreased from baseline of 51.4% to week 12 and of 59.1% to week 48 with a no significant change between weeks 12 and 48. After 12 weeks of follow-up was re- ported a decrease of 50% or more of seizures in 50% of patients and in 57% at 48 weeks. The safety results of the drug showed that of the 55 patients,10 patients withdrew by week 48, in- cluding 5 by weeks 12 and 48 due to lack of efficacy (n = 4) and AEs (n = 1). A total of 15 (27%) participants withdrew by week 144 of extended follow-up. There were no deaths during the study. SAEs that occurred during treatment were convulsions (9%), status epilepticus (9%) and respiratory infection (5%). While other adverse events reported more frequently were diar- rhea (29%), drowsiness (22%) and fatigue (22%). These results can demonstrate the safety and tolerability of long-term treatment with CBD and the reduction in seizure frequency in these four aetiologies of epilepsy.

Chen, et al. [88] conducted an open-label study, the aim was to assess the tolerability and safe- ty of CBD (Epidiolex) in the treatment of drug-resistant epilepsy in children. Sydney Chil- dren's Hospital Network Human Research Ethics Committee approved the protocol for this

study. Children (n = 40; mean age 8.5 years) with drug-resistant epilepsy and uncountable daily seizures in focal/multifocal epilepsy, epileptic encephalopathy, LGD and DS, were enrolled. CBD was administrated in addition to anti-epileptic therapy at the initial dose of 5 mg/Kg daily for 12 weeks. The initial dose was increased every week by 5 mg/Kg daily up to a maximum dose of 25 mg/Kg daily. During the treatment five children withdrew from the study, two be- cause he had an increase in the frequency of the seizures, one because has manifested signifi- cant somnolence, one for respiratory depression and one because their transaminase level was elevated. SAEs occurred in 15 out of patients, the frequent recurring to treatment were in- creased seizure number (in eight patients), intercurrent illness (in five patients), liver function disorder (in all patients), hyperlipidemia (in all patients), severe somnolence with anorexia and respiratory depression (in one patient). Over-therapeutic phenytoin levels are another SAEs manifested in two participants were considered related to treatment and occurred at doses of 10 mg/kg/day and 20 mg/kg/day. All participants showed AEs not all attributable to treatment. While, AEs that occurred frequently (15 individuals) and linked to the treatment was the drowsiness (AEs spontaneously resolved the 10 participants), and gastrointestinal disorders (nausea, vomiting, diarrhoea) in nine patients, somnolence (13 individuals) and increased seizures (two individuals). Instead, 12 children showed an improvement in health in general. The results reported in this study show that Epidiolex can be considered useful as adjuvant therapy. The presence of adverse events and possible interactions with antiepileptic drugs are important aspects to be taken into consideration.

Szaflarski, et al. [89] conducted an open-label, Expanded-Access Program (EAP) in 25 epilep- sy centers in the USA, and it was approved by an institutional review board at each site. The aim of this study was to evaluate the safety and efficacy of CBD oral solution (Epidiolex), in addition to common AEDs in patients with different forms of treatment-resistant epilepsies (TREs). For the study, 607 patients with a mean age of 13 were enrolled. All patients were in- cluded in the safety study, while 508 were included in the efficacy study. Treatment involved a 4-week baseline period followed by a 96-week treatment period. During treatment, patients received Epidiolex at the initial dose of 2–10 mg/Kg up to a maximum dose of 25-50 mg/Kg daily. 146 patients (mostly due to lack efficacy [15%] or AEs [5%]) from the safety study group and 136 patients (mostly due to lack efficacy [15%] or AEs [4%]) from the efficacy group were withdrawn from the study. SAEs were found in 33% of patients such as convulsion (9%), status epilepticus (7%), pneumonia (5%), and vomiting (3%). Instead, AEs manifested in 88% of pa- tients the most common were diarrhea (29%), somnolence (22%), and convulsion (17%). Al- ready after 12 weeks of treatment, the median monthly frequency of seizure convulsions was reduced by 51% and by 48 % the frequency of total seizures. These reductions remained stable during the 96 weeks of treatment. Between weeks 12 and 96 the average dose of CBD was 25 mg/kg daily, 55% of patients at follow-up had reduced the dose. Half of the patients taking concomitant CLB and valproate reduced the dose compared to baseline during the study. While, most of those who take simultaneously levetiracetam, had remained at their basal dos- es. The data obtained from this study show that CBD as an adjunct treatment to common AEDs can be used in the long-term effective treatment in patients with TRE.

A very interesting data in these studies is the pharmacokinetics and interaction of CBD with common AEDs. The interaction of these drugs is very complex and is linked to the individual metabolites produced and to the possible metabolic pathways that are involved. Specifically, the study conducted by Geffrey, et al. [72] shows a bidirectional drug-drug interaction when CBD is administered with the CLB for long period of time. Therefore, CLB determines an in- crease in serum levels of the CBD metabolite (7-OH-CBD) and conversely, CBD causes an increase in the metabolite of CLB (n-CLB). CBD is an inhibitor of CYP2C19, an enzyme in- volved in the degradation of n-CLB, these explain how the CBD associated with CLB causes elevated plasma levels of this metabolite. In contrast, CBD does not influence the pharmacoki- netics of valproate and stiripentol when co-administered, moreover, stiripentol causes a slight decrease in CBD metabolites (7-OH-CBD 7-COOH-CBD) while valproate causes a slight in- crease of 7-OH-CBD. The mechanisms by which these interactions take place are not yet known but do not cause clinically relevant effects. However, the CBD shows good safety pro- files, the interaction with these two drugs does not require the interruption of therapy. The modulation of the dose of these AEDs will be sufficient to resolve the adverse events. In con- clusion, the results of these studies show that the administration of CBD as an addition to the common AEDs for long periods of time leads to clinically significant reductions in the fre- quency of convulsive and total seizures in different etiologies of epilepsy. Furthermore, an im-provement in the quality of life of these patients was also observed.

6. Conclusions

The CBD is a compound extensively studied for its potential efficacy for the treatment of epilepsy. In this review, we reported the studies conducted in infants, children and teenagers affected by epilepsy resistant to common AEDs.

To date, available safety data show that the administration of CBD associated with other AEDs causes non-serious adverse events, which can be resolved reducing the dose of CBD and/or common AEDs. In this context, particular attention should be paid when CBD is associated with valproate and CLB. Specifically, abnormal liver function was noted in participants taking concomitant valproate, therefore, it is necessary to monitor serum levels of these compounds and their respective metabolites. Instead, when CBD is associated with CLB it induces an in- crease in its metabolites. Since the adverse effects are not serious, this association can be con- sidered safe. The available results also highlight the efficacy of CBD as adjunctive to common AEDs. The mechanism by which CBD interacts with other AEDs is not yet fully known, as many meta- bolic pathways involved in this interaction are still unknown. In addition, not all the molecular targets used by the CBD to exercise its antiepileptic action are yet known. However, the results obtained to date encourage the use of CBD associated with AEDs.

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Author Contributions

S.S. wrote the manuscript; S.M. contributed to bibliographic research; E.C. contributed in graphical support; P.B. and E.M. revised the manuscript.

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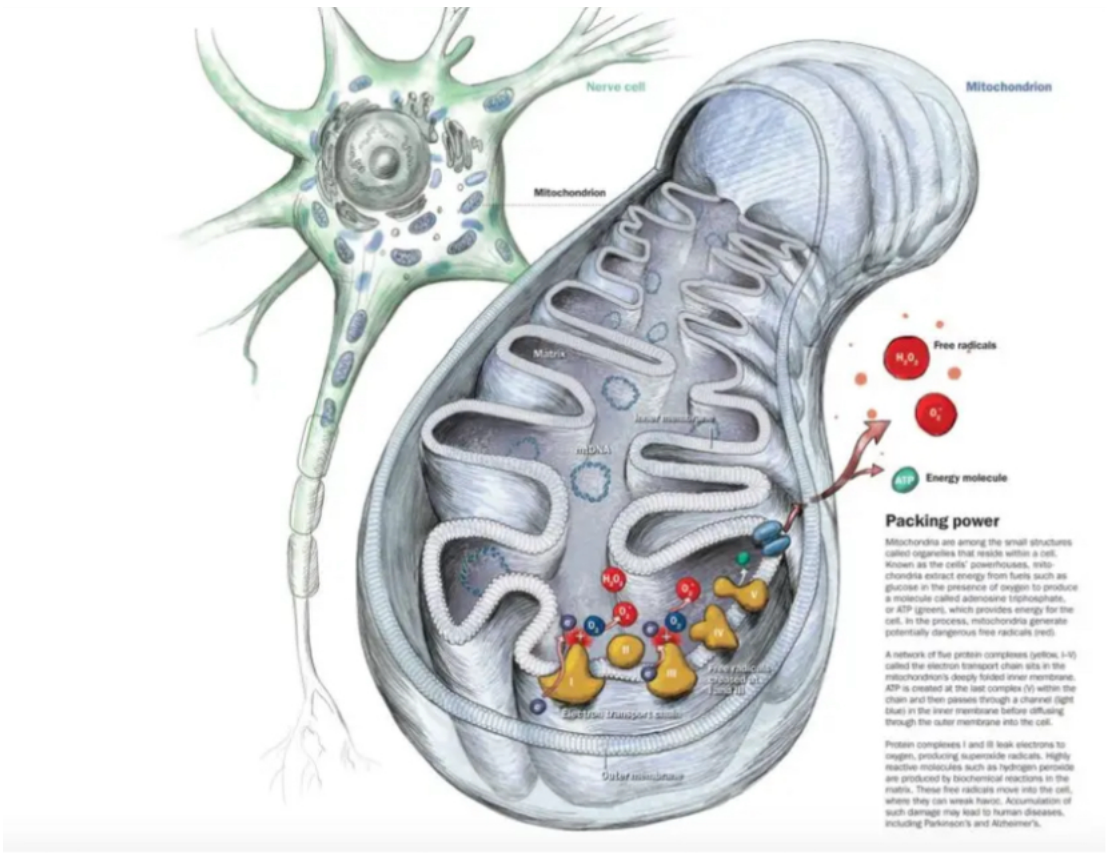
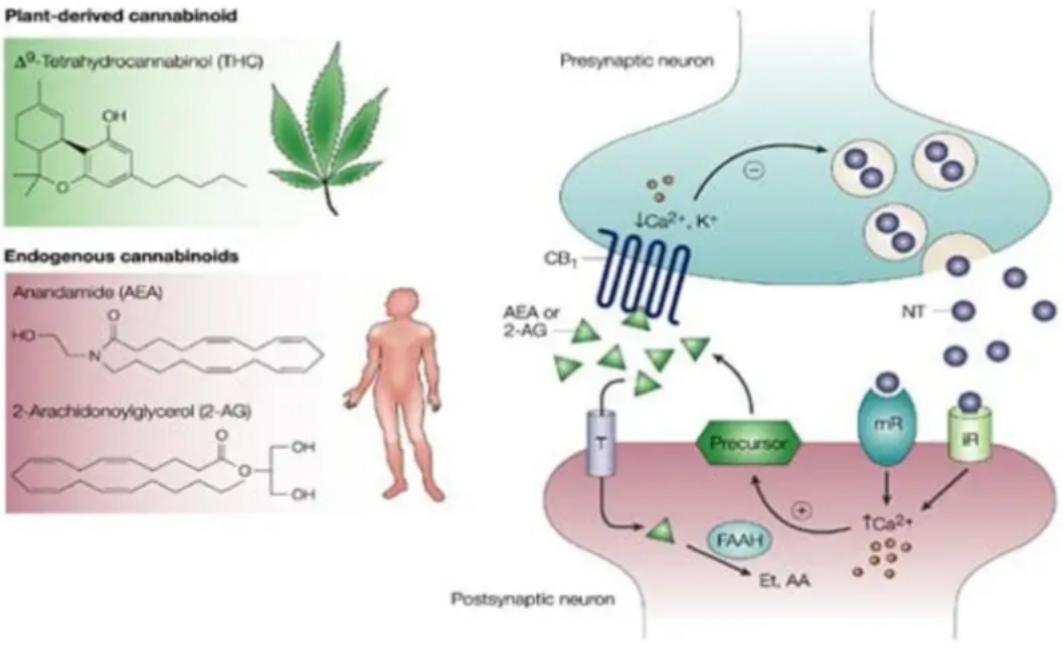
Conflicts of Interest

The authors declare no conflict of interest.


Nerve cell
Mitochondrion
Mitochondrion
Matrix
mtDNA
Free radicals
Energy molecule
Packing power
Mitochondria are among the small structures called organelles that reside within a cell. Known as the cells' powerhouses, mitochondria extract energy from fuels such as glucose in the presence of oxygen to produce a molecule called adenosine triphosphate, or ATP (green), which provides energy for the cell. In the process, mitochondria generate potentially dangerous free radicals (red).
A network of five protein complexes (yellow, I–V) called the electron transport chain sits in the mitochondrion's deeply folded inner membrane. ATP is created at the last complex (V) within the chain and then passes through a channel (light blue) in the inner membrane before diffusing through the outer membrane into the cell.
Protein complexes I and III leak electrons to oxygen, producing superoxide radicals. Highly reactive molecules such as hydrogen peroxide are produced by biochemical reactions in the matrix. These free radicals move into the cell, where they can wreak havoc. Accumulation of such damage may lead to human diseases, including Parkinson's and Alzheimer's.



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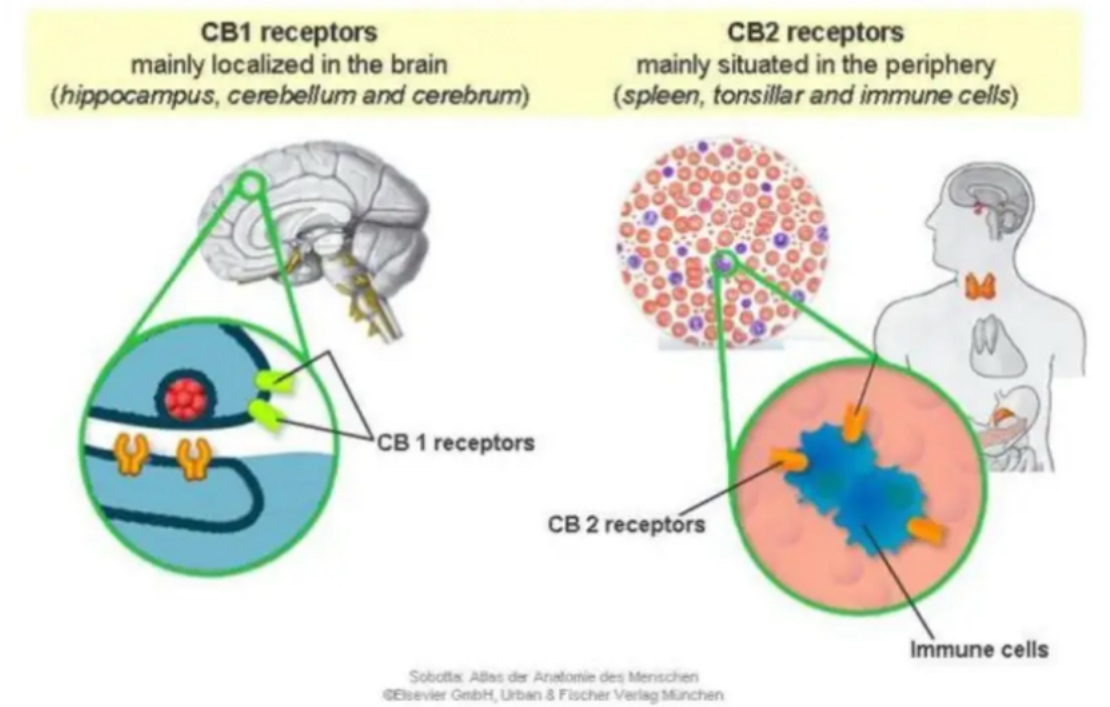
CB1 receptors
mainly localized in the brain
(hippocampus, cerebellum and cerebrum)
CB2 receptors
mainly situated in the periphery
(spleen, tonsillar and immune cells)
CB 1 receptors
CB 2 receptors
Immune cells
Sobotta: Atlas der Anatomie des Menschen
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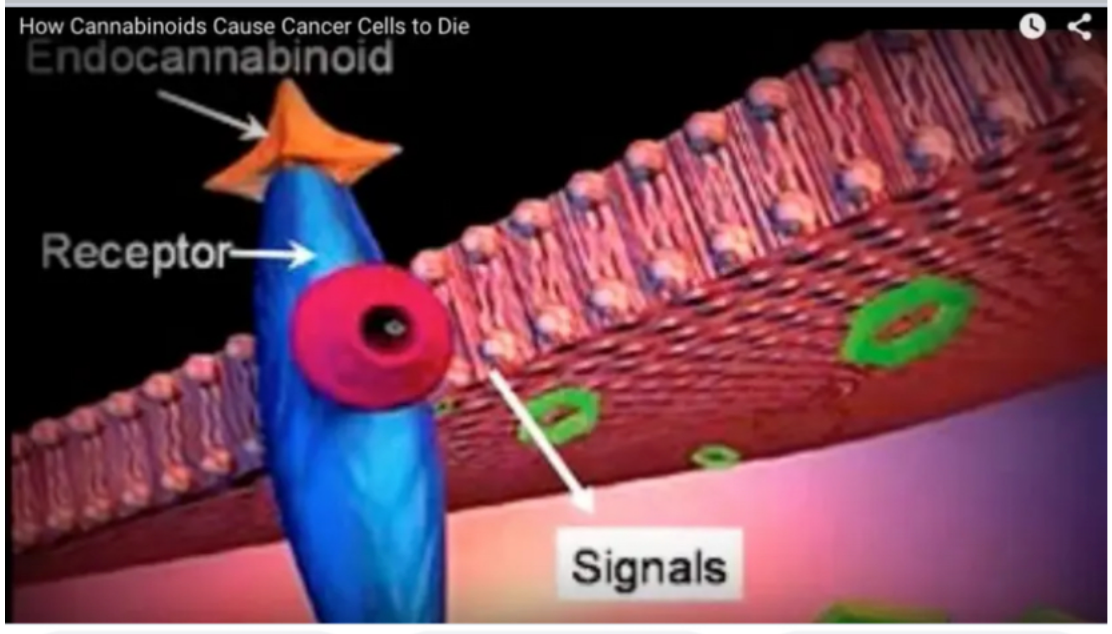

The Biochemist Who Cured His Cancer With Cannabis Oil

SEPTEMBER 17, 2015 BY LUKE SUMPTER 30 COMMENTS

THC starts off in the plant as Geranyl Py' °o Phosphate & Olivetolic acid through an enzyme catilized reaction. The Geranyl Py' °o Phosphate & Olivetolic acid are catilized to Cannabigerolic acid CBGA, CBGA is the precurser to several cannabinoids, to make THC Geranyl Py' °o Phosphate & Olivetolic acid gose through the pathway catalized by another enzyme called THC
Synthase, it allowes the CBGA to become Tetrahydrocannabinol carboxylic acid THCA, THCA has vary different effects than THC, now with heat or over time THCA is decarboxylized to free THC then to Cannabinol CBN, Carboxylic acid atached to the end of THC inside of fresh Cannabis renders this compound virtualy non-psycheactive cannabinoids such as THCA, THC CBDA, CBD THCV, CBGA attaches to cannabinoid receptors on cancer cells which has 3-12 or more receptors vs. 2 (CB1) (CB2) in normal cells in our body & translocate bad mitochondria cancer cells to produce RNA which disintegrates, dissolves the protein walls of the endoplasm reticulum while hijacking the lipoxygenase nutrient energy pathway & Cellular Lysosome, the cells digestive system to trigger cellular self cannibalism, apoptosis leaving no possibility at all of cancer cell survival.
THC is a natural fit for the CB1 cannabinoid receptor on the cancer cell surface. When THC hits the receptor, the cell generates ceramide that disrupts the mitochondria, closing off energy for the cell. Disruption of the mitochondria releases cytochrome c and reactive oxygen species into the cytosol, hastening cell death
If ceramide (a signaling metabolite of sphingosine-1-phosphate) is high, then cell death (apoptosis) is imminent. If ceramide is low, the cell will be strong in its vitality.
The primary job of CBD in the cancer cell is to disrupt the endoplasmic reticulum through the wrecking of the calcium metabolism, pushing calcium into the cytosol. This always results in cell death. Another pathway for CBD to effect cancer cell death is the caspase cascade, which breaks down proteins and peptides in the cell.
When this happens the cell cannot survive.

Vast amounts of people are coming forward to testify as to how they have treated - and in some cases completely cured - serious diseases with cannabis extracts. These personal testimonials even cover diseases that the medical establishment often fails to effectively treat, such as cancer.

Despite a deficiency in human trials, there are a significant amount of studies that show compounds found within cannabis, known as cannabinoids, have anti-cancer properties. The United States' federally funded National Institute of Health even goes as far as to state on their website that: "Cannabinoids may cause antitumor effects by various mechanisms, including induction of cell death, inhibition of cell growth, and inhibition of tumor angiogenesis invasion and metastasis."

To gain both a greater personal and scientific insight into the cancer-fighting prowess of cannabis, Reset spoke to Dennis Hill who, having earned a degree in biochemistry from the University of Houston, has worked in medical research at the University of Texas' M.D. Anderson Cancer Center. Hill currently resides in California, where he now works in software engineering.

Photo: Dennis Hill

Following a biopsy in 2010, Hill was diagnosed with aggressive Stage 4 adenocarcinoma of the prostate, with Gleason scores of 7s and 8s. A digital exam also revealed metastatic lesions in the bladder and rectum.

We asked Hill what approach he took to treating the disease. "Opting out of radiation, chemo and surgery, I chose cannabis extract," replies Hill. "Within six months the cancer was in remission."

How did Hill achieve this truly staggering result? "I never really noticed any dramatic changes; the cancer just went away, he tells us. "After three months, the primary tumor was gone; after three more months, the metastatic lesions were gone.

So how exactly does cannabis work in the body? Hill, utilizing his expert knowledge of human biochemistry, explains to us in detail exactly what occurs when a cancer patient ingests cannabinoids:

"Let us look first at tetrahydrocannabinol (THC) and observe that THC is a natural fit for the CB1 cannabinoid receptor on the cancer cell surface. When THC hits the receptor, the cell generates ceramide that disrupts the mitochondria, closing off energy for the cell. Disruption of the mitochondria releases cytochrome c and reactive oxygen species into the cytosol, hastening cell death. It is notable that this process is specific to cancer cells. Healthy cells have no reaction to THC at the CB1 receptor. The increase in ceramide also disrupts calcium metabolism in the mitochondria, completing the demise to cell death.

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"The other cannabinoid we know is effective in killing cancer cells is cannabidiol (CBD). The primary job of CBD in the cancer cell is to disrupt the endoplasmic reticulum through the wrecking of the calcium metabolism, pushing calcium into the cytosol. This always results in cell death. Another pathway for CBD to effect cancer cell death is the caspase cascade, which breaks down proteins and peptides in the cell. When this happens the cell cannot survive. Again, these processes are specific to cancer cells, no normal cells are affected."

Hill goes on to explain how certain cannabinoids destroy cancerous tumors by working symbiotically with our body's endocannabinoid system:

"The endocannabinoid system (ECS) started revealing itself to researchers in the 1940s and by the late '60s the basic structure and functionality had been laid out.

Today we know the ECS is a comprehensive system of biochemical modulators that maintain homeostasis in all body systems including the central and peripheral nervous systems, all organ systems, somatic tissues, and all metabolic biochemical systems, including the immune system.

"This homeostatic matrix is not a recent evolutionary twist just for humans; we find the endocannabinoid system in every chordate creature for the last 500 million years. It is a fully mature biochemical technology that has maintained health and metabolic balance for most of the history of life itself.

endocannabinoid-natur

"The two major interactive systems within the ECS are one, the cannabinoid receptors that we find on all cell surfaces and neurological junctions and two, the endocannabinoids that fit the receptors to trigger various metabolic processes. Looking at a cannabinoid receptor distribution map we see that CB1 receptors, that are most sensitive to anandamide, are found in the brain, spinal nerves, and peripheral nerves. CB2 receptors preferred by 2-Arachidonoylglycerol (2-AG) are found largely in the immune system, primarily the spleen. A mix of CB1 and CB2 receptors are found throughout the rest of the body including the skeletal system. And yes, 2-AG or CBD will grow new trabecular bone. It is also useful to note that both anandamide and 2-AG can activate either CB1 or CB2 receptors.

"The nature of the endocannabinoids are functionally much like neurotransmitters, but structurally are eicosanoids in the family of signaling sphingolipids.

These signaling cannabinoids keep track of metabolic systems all over the body. This information is shared with the nervous system and the immune system so that any imbalance is attended to. If the body is in chronic disease or emotional stress, the immune system can fall behind and lose control of compromised systems. It is here that

phytocannabinoids can pitch in to support the stressed body in a return to health. The cannabis plant provides analogues of the body's primary signaling cannabinoids. Tetrahydrocannabinol (THC) is mimetic to anandamide, and cannabidiol (CBD) is mimetic to 2-AG, and has the same affinity to CB1 and CB2 receptors, providing the body with additional support for the immune and endocannabinoid systems.

"Phytocannabinoids supercharge the body's own endocannabinoid system by amping up the response to demand from the immune signaling system in two modes of intervention: one, of course, is in bonding with the cannabinoid receptors; the other is in regulation of innumerable physiological processes, such as cannabinoid's powerful neuroprotective and anti-inflammatory actions, quite apart from the receptor system. It is interesting to note here that the phytocannabinoids and related endocannabinoids are functionally similar, but structurally different. As noted above, anandamide and 2-AG are eicosanoids while THC and CBD are tricyclic terpenes.

"The National Institutes of Health tells us that THC is the best known because of its signature psychotropic effect. This government report shows THC to be effective as an anticancer treatment, an appetite stimulant, analgesic, antiemetic, anxiolytic, and sedative. There is a plentiful supply of research articles and personal testaments that show the efficacy of cannabis effecting cancer remission. However, only a few point to the mechanism by which the cancer cells die. To understand this better we need to know what metabolic processes provide life to the cells.
"There are two structures in most cells that sustains life one is the mitochondria, and the other is the endoplasmic reticulum. The mitochondria primarily produce adenosine triphosphate (ATP) that provides the necessary energy. The endoplasmic reticulum (ER) is a loosely bound envelope around the cell nucleus that synthesizes metabolites and proteins directed by the nuclear DNA that nourish and sustain the cell..

THC starts off in the plant as Geranyl Py' °o Phosphate & Olivetolic acid through an enzyme catilized reaction. The Geranyl Py' °o Phosphate & Olivetolic acid are catilized to Cannabigerolic acid CBGA, CBGA is the precurser to several cannabinoids, to make THC Geranyl Py' °o Phosphate & Olivetolic acid gose through the pathway catalized by another enzyme called THC
Synthase, it allowes the CBGA to become Tetrahydrocannabinol carboxylic acid THCA, THCA has vary different effects than THC, now with heat or over time THCA is decarboxylized to free THC then to Cannabinol CBN, Carboxylic acid atached to the end of THC inside of fresh Cannabis renders this compound virtualy non-psycheactive cannabinoids such as THCA, THC CBDA, CBD THCV attaches to cannabinoid receptors on cancer cells which has 3-12 or more receptors vs. 2 (CB1) (CB2) in normal cells in our body & translocate bad mitochondria cancer cells to produce RNA which disintegrates, dissolves the protein walls of the endoplasm reticulum while hijacking the lipoxygenase nutrient energy pathway & Cellular Lysosome, the cells digestive system to trigger cellular self cannibalism, apoptosis leaving no possibility at all of cancer cell survival.

CB1and2_Crop
"In every cell there is a family of interconvertible sphingolipids that specifically manage the life and death of that cell. This profile of factors is called the
'Sphingolipid Rheostat.' If ceramide (a signaling metabolite of sphingosine-1-phosphate) is high, then cell death (apoptosis) is imminent. If ceramide is low, the cell will be strong in its vitality. Very simply, when THC connects to the CB1 or CB2 cannabinoid receptor site on the cancer cell, it causes an increase in ceramide synthesis which drives cell death. A normal healthy cell does not produce ceramide in the presence of THC, thus is not affected by the cannabinoid.
"The cancer cell dies, not because of cytotoxic chemicals, but because of a tiny little shift in the mitochondria. Within most cells there is a cell nucleus, numerous mitochondria (hundreds to thousands), and various other organelles in the cytoplasm. The purpose of the mitochondria is to produce energy (ATP for cell use. As ceramide starts to accumulate, turning up the Sphingolipid Rheostat, it increases the mitochondrial membrane pore permeability to cytochrome c, a critical protein in energy synthesis. Cytochrome c is pushed out of the mitochondria, killing the source of energy for the cell.
"Ceramide also causes genotoxic stress in the cancer cell nucleus generating a protein called p53, whose job it is to disrupt calcium metabolism in the mitochondria.
If this weren't enough, ceramide disrupts the cellular lysosome, the cell's digestive system that provides nutrients for all cell functions. Ceramide, and other sphingolipids, actively inhibit pro-survival pathways in the cell leaving no possibility at all of cancer cell survival.
"The key to this process is the accumulation of ceramide in the system. This means taking therapeutic amounts of cannabinoid extract, steadily, over a period of time, keeping metabolic pressure on this cancer cell death pathway.
"How did this pathway come to be? Why is it that the body can take a simple plant enzyme and use it for healing in many different physiological systems? This endocannabinoid system exists in all animal life, just waiting for it's matched exocannabinoid activator.
"This is interesting. Our own endocannabinoid system covers all cells and nerves; it is the messenger of information flowing between our immune system and the central nervous system (CNS). It is responsible for neuroprotection, and micro-manages the immune system. This is the primary control system that maintains homeostasis: our wellbeing..
"Endocannabinoids have their origin in nerve cells right at the synapse. When the body is compromised through illness or injury it calls insistently to the endocannabinoid system and directs the immune system to bring healing. If these homeostatic systems are weakened, it should be no surprise that exocannabinoids perform the same function. It helps the body in the most natural way possible.
"To see how this works we visualize the cannabinoid as a three dimensional molecule, where one part of the molecule is configured to fit the nerve or immune cell receptor site just like a key in a lock. There are at least two types of cannabinoid receptor sites, CB1 (CNS) and CB2 (immune). In general CB1 activates the CNS messaging system, and CB2 activates the immune system, but it's much more complex than this. Both THC and anandamide activate both receptor sites.
Other cannabinoids activate one or the other receptor sites. Among the strains of cannabis, C. sativa tends toward the CB1 receptor, and C. indica tends toward CB2. So sativa is more neuroactive, and indica is more immunoactive. Another factor here is that sativa is dominated by THC cannabinoids, and indica can be higher in CBD (cannabidiol). "It is known that THC and CBD are biomimetic to anandamide and AG-2, that is, the body can use both interchangeably. Thus, when stress, injury, or illness demand more from endogenous anandamide than can be produced by the body, its mimetic exocannabinoids are activated. If the stress is transitory, then the treatment can be transitory. If the demand is sustained, such as in cancer, then treatment needs to provide sustained pressure of the modulating agent on the homeostatic systems.
"Typically CBD gravitates to the 5-HT1A and vanilloid receptors. CBD stimulates production of anandamide and AG-2, endogenous cannabinoids that are agonists for CB-1 and CB-2 receptors. From there, immune cells seek out and destroy cancer cells. Interestingly, it has

been shown that THC and CBD cannabinoids have the ability to kill cancer cells directly without going through immune intermediaries. THC and CBD hijack the lipoxygenase pathway to directly inhibit tumor growth.
As a side note, it has been discovered that CBD inhibits anandamide re-uptake. Here we see that cannabidiol helps the body preserve its own natural endocannabinoid by inhibiting the enzyme that breaks down anandamide.
Screen Shot 2015-09-14 at 12.31.51 PM
"In 2006, researchers in Italy showed the specifics of how cannabidiol (CBD) kills cancer. CBD stimulates what is known as the Caspase Cascade, that kills the cancer cell. First, let's look at the nomenclature, then to how Caspase kills cancer. Caspase in an aggregate term for all cysteine-aspartic proteases. The protease part of this term comes from prote (from protein) and - ase (destroyer). Thus the caspases break down proteins and peptides in the moribund cell. This becomes obvious when we see caspase-3 referred to as the executioner. In the pathway of apoptosis, other caspases are brought in to complete the cascade.
"Even when the cascade is done and all the cancer is gone, CBD is still at work healing the body. Cannabidiol also shuts down the Id-1 gene; a gene that allows metastatic lesions to form. Fundamentally this means that treatment with cannabinoids not only kills cancer through numerous simultaneous pathways, but prevents metastasis. What's not to like... Nature has designed the perfect medicine that fits exactly with our own immune system of receptors and signaling metabolites to provide rapid and complete immune response for systemic integrity and metabolic homeostasis."
Finally, Reset asked what advice Hill would dispense to somebody with terminal cancer who is looking into the possibility of utilizing cannabis oil. He replies, "Many who have sought out cannabis as a last resort have recovered. It's never too late.'
Below is a video of Dennis Hill explaining how cannabinoids cause cancer cells to die:

Several derivatives of cannabinol and the 1,1-dimethylheptyl homolog (DMH) 3230 Journal of Medicinal Chemistry
Figure1. Compound 4a antagonizes the capacity of the cannabinoid
Cannabinol Derivatives: Binding to Cannabinoid Receptors and Inhibition of Adenylylcyclase
Several derivatives of cannabinol and the 1,1-dimethylheptyl homolog (DMH) of cannabinol were prepared and assayed for binding to the brain and the peripheral cannabinoid receptors(CB 1 and CB 2), as well as for activation of CB 1 -and CB 2 -mediated inhibition of adenylylcyclase. The DMH derivatives were much more potent than the pentyl (i.e., cannabinol) derivatives.11-Hydroxycannabinol (4a) was found to bind potently to both CB 1 and CB 2 (Ki values of 38.0 (7.2 and 26.6 (5.5 nM, respectively) and to inhibit CB 1 -mediated adenylylcyclase with an EC 50 of 58.1 (6.2 nM but to cause only 20% inhibition of CB 2 -mediated adenylylcyclase at 10 μ M. It behaves as a specific, though not potent, CB 2 antagonist. 11- Hydroxycannabinol-DMH(4b) is a very potent agonist for both CB 1 and CB 2 (Ki values of 100 (50 and 200 (40 pM;EC 50 of adenylylcyclase inhibition 56.2 (4.2 and 207.5 (27.8 pM, respectively).
Chart 1
Introduction
.Two cannabinoid receptors have been described to date. Both are proteins with seven transmembrane-spanning domains. These receptors were originally found in rat brain and spleen, respectively, and are generally known as the central cannabinoid receptor, CB 1, and the peripheral cannabinoid receptor, CB 2. 1,2 CB 1 is mainly expressed in the central nervous system(CNS). It is also found, to a lesser extent, outside the CNS, in numerous other tissues such as vas deferens, adrenal gland, heart, lung, prostate, uterus, ovary, testis, bone marrow, thymus, and tonsils. 3,4 The CB 2 gene is not expressed in the brain, being found mostly in the immune system. In certain tissues, such as spleen, the mRNA content of CB 2 is particularly high. In blood cell subpopulations, it is found particularly in B-cells. 3,4 It has been suggested that “cannabinoids may exert specific receptor-mediated action on the immune system through the CB 2 receptor”. 4 While a considerable amount of work has been done on structure - activity relationships (SAR) as regards binding to CB 1, 5 - 7 very little is known

on binding to CB 2. 8 In the original paper on the identification of CB 2 ,Munro et al. 9 showed that cannabinol (CBN) (1a), which only binds feebly to CB 1, much less so than Δ 9 -tetrahydrocannabinol (Δ 9 - THC) (2a), binds to CB 2 at the samelevel of potency as Δ 9 -THC. The low binding to CB 1 is compatible with the low THC-type in vivo activity recorded for CBN when compared to Δ 9 -THC. 10 The binding of CBN to CB 2, with a potency equivalent to that of Δ 9 -THC, suggests that cannabinol derivatives could serve as a novel starting point for SAR analysisin the cannabinoid series and could possibly lead toselectivity as regards binding to the two cannabinoid receptors. We present here data on several cannabinol derivatives which have been tested for binding to. CB 1 and CB 2. Both CB 1 and CB 2 binding assays were performed with transfected cells. 11 - 13 For comparison purposes, the derivatives were also assayed for CB 1 binding to membranes, as previously described. 14 Various laboratories, including our own, have established that the signal transduction pathway utilized by the two cannabinoid receptors proceeds through pertussis toxin-sensitive G proteins and inhibition of adenylylcyclase activity. 2 We recently showed that Δ 9 -THC binds to both receptors with similar affinity; however, in contrast to its capacity to serve as an agonist for the CB 1 receptor and to inhibit CB 1 -mediated adenylylcyclase, Δ 9 -THC was only able to induce a very slight inhibition of adenylylcyclase at the CB 2 receptor. 12 *

Address correspondence to this author. Fax: 972-2-6410740.E-mail: mechou@yam-suff.cc.huji.ac.il. † The Weizmann Institute of Science. ‡ Hebrew University. X.Abstract published in Advance ACS Abstracts, August 15, 1997. Chart 1 3228 J. Med. Chem. 1997, 40, 3228 - 3233S0022- 2623(97)00126-X CCC: $14.00 © 1997 American Chemical Society

Cannabinol Derivatives Scheme 1

Morever Δ 9-THC antagonizes the agonist induced inhibition of adenylylcyclase mediated by CB 2. Therefore,we concluded that Δ 9-THC constitutes a weak antagonist for the CB 2 receptor. 12 It was thus of interest to find out whether CBN derivatives could selectively activate one of the cannabinoid receptors. Therefore, all compounds were tested for CB 1 -as well as CB 2 -mediated inhibition of adenylylcyclase. The present paper is apparently the first one which examines the SAR of cannabinol derivatives for binding to the cannabinoid receptors as well as for inhibition of adenylylcyclase.

Chemistry

Cannabinol (1a), Δ 9 -THC (2a), and cannabidiol (3a) were extracted from hashish 15,16 The 1,1-dimethylhep-tyl (DMH) homolog (2c) (at position 3) of Δ 9 -THC was prepared by Lewis acid-cata lyzed ring cyclization of the DMH homolog of cannabidiol (3b), 17 as previously described for the parallel conversion of cannabidiol (3a) into Δ 9 -THC (2a). 16 The DMH homolog of cannabinol (1b) was prepared by dehydrogenation with sulfur at 240 °C of 2d (synthesized by acetylation of 2c), leading to 1c, followed by removal of the acetate group with ethanolic sodium hydroxide. The isomeric 1,2-dimeth-ylheptyl homolog of CBN has previously been prepared and shown to be a potent cannabimimetic. 18 11-Hy-droxycannabinol (4a) was prepared, as previously described, by selenium dioxide oxidation and dehydrogenation of Δ 9 -THC acetate (2b) followed by removal of the ester grouping. 19 11-Hydroxy- Δ 8 -THC (5c) and its DMH homolog (5a) were prepared as previously described. 19,20 Dehydrogenation of the DMH homolog of11-hydroxy- Δ 8 -THC diacetate (5b) 20 with sulfur at 240°C led to the DMH homolog of 11-hydroxycannabinol diacetate (4c), which was converted to the diol 4b. The same procedure was followed for the conversion of Δ 8 -THC-7-oic acid (6c) 21 into cannabinol-7-oic acid
(7a) 22 and of the DMH homolog of (-) - Δ 8 -THC-11-oic acid (6a) 23 into the DMH homolog of cannabinol-11-oic acid (7c). The lactones 8a, b were prepared following aroute reported by the groups of Adams and Todd about 50 years ago. 24 Von Pechmann condensation of eitherolivetol (9a) or 3-(1,1-dimethylheptyl) resorcinol (9b) with ethyl 5-methyl-1-oxocyclohexane-2 carboxylate (10) led to the known tetrahydrodibenzopyrone 11a or 11b , respectively. 24 Dehydrogenation of 11a or 11b asdescribed above gave 8a 24 or 8b, respectively.

Biological Results

All compounds were tested for binding to CB 1 using African green monkey kidney (COS-7) cells transfected with the cDNA of rat CB 112,25 as well as rat brain synaptosomal membrane preparations. 14,26 Binding to CB 2 was performed using transfected COS-7 and Chi-nese hamster ovary (CHO) cells transfected with the cDNA of human CB 2. 9,11,12 The Ki values were deter-mined by displacement of [3 H] HU-243, a probe for both cannabinoid receptors. 26,27 For consistency, the results discussed below are those from transfected cells except when specifically indicated. All compounds were also assayed for inhibition of CB 1-and CB 2-mediated ade-nylylcyclase. 11,12 The results are summarized in Table1.First, CBN (1a) was compared to Δ 9-THC (2a). Underour experimental conditions, CBN was about 2-4 timesless potent on binding to either CB 1 or CB 2, in contrastto the data reported by Munro et al., 9 who found that CBN and Δ 9-THC are equipotent on CB 2. These differ-ences in recorded potencies are not unexpected, asbinding data are very sensitive to experimental condi-tions. The CB 1-mediated adenylylcyclase inhibitiondata seem to be closer to in vivo observations, where,as mentioned above, Δ 9-THC is considerably morepotent than CBN. We found that CBN is about 13 timesless potent than Δ 9-THC. With CB 2 we see a different profile. While the EC 50 of CBN inhibition of CB 2-mediated cyclase is 261.2 ± 46.4 nM, Δ 9- THC causesonly 21% inhibition at 1 μM. We have previously shownthat Δ 9-THC is a weak agonist for CB 2 and can actuallyantagonize the CB 2- mediated inhibition of adenylylcy-clase caused by more potent agonists. 12 The next derivative to be examined was the DMH homolog of CBN (1b). We observed essentially nodifference between its binding affinity to CB 1 or CB 2. On both receptors, 1b was about 100 times more potent than CBN. It was also 100 times more potent than CBN when tested for binding to rat brain membranes. This compound also potently inhibited adenylylcyclase via both CB 1 and CB 2: the Ki of inhibition by 1b of CB 1 -mediated cyclase was ca. 660-fold lower, and the CB 2-mediated cyclase Ki was ca. 300-fold lower than that of CBN. This result demonstrates that the exchange ofthe pentyl group with the DMH group (at position 3) dramatically increases the affinity of the ligand to bothCB 1 and CB 2 and makes it a much more potent agonist.A similar result was obtained with other cannabinol derivatives (see below). Neither CBN (1a) nor its DMH homolog 1b express any particular selectivity with regard to the inhibition of adenylyl cylase via the two receptors. However, a major metabolite of CBN, namely,11-hydroxy-CBN (4a), 19,22,28 had a different profile. It binds to both receptors with Ki values ca. 5-fold lowerthan the Ki values recorded for CBN. However, while 3230 Journal of Medicinal Chemistry, 1997, Vol. 40, No. 20

Table1.
Binding of Various Cannabinoids to Cannabinoid Receptors and Inhibition of Adenylylcyclase a,b compound
brain synaptosomal binding, (CB 1 (Ki, nM)
binding (Ki, nM)
COS CB 1 and CB 2
CB 1
CB 2
adenylylcyclase (EC 50, nM)
CB 1
CB 2
compound
CBN (1a)
brain synaptosomal binding, (CB 1 (Ki, nM)
392.2 ± 53. 5
COS
CB 1 and CB 2 binding (Ki, nM)
CB 1
211.2 ± 35.0
CB 2
126.4 ± 26.0
adenylylcyclase (EC 50, nM)
CB 1
120.0 ± 32.1
CB 2
261.2 ± 46.4
compound
CBN-DMH (1b)
brain synaptosomal binding, CB 1 (K1, nM)
3.3 ± 0.2
COS CB 1 and CB 2 binding (Ki, nM)
CB 1
2.0 ± 0.3
CB 2
1.5 ± 0.5
adenylylcyclase (EC 50, nM)
CB 1
0.18 ± 0.03
CB 2
0.79 ± 0.021
compound
Δ 9 THC (2a)
brain synaptosomal binding, CB 1 (K1, nM)
66.5 ± 5.8
COS CB 1 and CB 2 binding (Ki, nM)
CB 1
80.3 ± 22.2
CB2 32.2 ± 6.7
adenylylcyclase (EC 50, nM)
CB 1
11.0 ± 2.1
CB 2
21% inhib at 1 μM
compound
Δ 9 - THC-DMH (2c)
brain synaptosomal binding, CB 1 (K1, nM) 2.6 ± 0.3
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
0.241 ± 0.05
CB 2

0.199 ± 0.05
adenylylcyclase (EC 50, nM)
CB 1
0.58 ± 0.12
CB 2
1.01 ± 0.15
compound
11-OH-CBN (4a)
brain synaptosomal binding, CB 1 (K1, nM) 188.9 ± 38.3
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
38.0 ± 7.2
CB 2
26.6 ± 5.5
adenylylcyclase (EC 50, nM)
CB 1
58.1 ± 6.2
CB 2
20% inhib at 10 μM
compound
11-OH-CBN-DMH (4b)
brain synaptosomal binding, CB 1 (K1, nM) 1.8 ± 0.3
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
0.1 ± 0.05
CB 2
0.2 ± 0.04
adenylylcyclase (EC 50, nM)
CB 1
0.056 ± 0.004
CB 2
0.208 ± 0.028
compound
11-OH-Δ 8-THC (5c)
brain synaptosomal binding, CB 1 (K1, nM) 33.4 ± 3.8
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
52.8 ± 2.9
CB 2
7.4 ± 2.5
adenylylcyclase (EC 50, nM)
CB 1
39.9 ± 8.9
CB 2
19% inhib at 1 μM
compound
11-0H-Δ 8- THC-DMH (5a)
brain synaptosomal binding, CB 1 (K1, nM) 0.19 ± 0.01
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
0.1 ± 0.02
0.17 ± 0.01
CB 2
adenylylcyclase (EC 50, nM)
CB 1
0.035 ± 0.005

CB 2
0.078 ± 0.009
compound
Δ 8-THC-11-oic acid-DMH (6a)
brain synaptosomal binding, CB 1 (K1, nM)
480.6 ± 40.8
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
32.3 ± 3.7
CB 2
170.5 ± 7.8
adenylylcyclase (EC 50, nM)
CB 1
927.0 ± 39.6
CB 2
116.2 ± 74.7
compound
Δ 8-THC-11-oic acid-DMH
brain synaptosomal binding, CB 1 (K1, nM) >1 0000
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
11% in hib at 1 μM
CB2
9% inhib at 1 μM
adenylylcyclase (EC 50, nM)
CB 1
no inhib at 1 μM
CB 2
27% inhib at 1 μM
compound
acetate (6b)
compound
CBN-11oic- acid- (7a)
brain synaptosomal binding, CB 1 (K1, nM) 602.4 ± 15.8
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
<2 % inhib at 1 μM
CB 2
23% inhib at 1 μM
adenylylcyclase (EC 50, nM)
CB 1
10% inhib at 1 μM
CB2
6% inhib at 1 μM
compound
CBN-11-oic acid acetate (7b)
brain synaptosomal binding, CB 1 (K1, nM) >1 0000
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
35% inhib at 1 μM
CB 2
19% inhib at 1 μM
adenylylcyclase (EC 50, nM)
CB 1
3 inhib at 1 μM
CB 2

2% inhib at 1 μM
compound
CBN-DMH-11-oic acid (7c)
brain synaptosomal binding, CB 1 (K1, nM) 6.1 ± 0.7
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
6.1 ± 1.1
CB 2
4.8 ± 1.9
adenylylcyclase (EC 50, nM)
CB 1
2.4 ± 0.2
CB 2
5.4 ± 0.3
compound
CBN-DMH-11-oic acid
brain synaptosomal binding, CB 1 (K1, nM) 556.1
COS CB 1 and CB 2
binding (Ki, nM) CB 1
49.8 ± 11.5
CB 2
17.3 ± 7.4
adenylylcyclase (EC 50, nM)
CB 1
13.3 ± 3.4
CB 2
39.3 ± 6.1
compound
acetate (7d)
compound
lactone 8a
brain synaptosomal binding, CB 1 (K1, nM)
>1 0000
COS CB 1 and CB 2
binding (Ki, nM)
14% inhib at 1 μM
adenylylcyclase (EC 50, nM)
CB 1
7% inhib at 1 μM
CB 2
no inhib at 1 μM
compound
lactone 8a
brain synaptosomal binding, CB 1 (K1, nM)
72.5 ± 3.6
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
32.7 ± 4.9
CB 2
17.3 ± 5.6
adenylylcyclase (EC 50, nM)
CB 1
9.6 ± 0.6
CB 2
4.6 ± 0.3
a For details of procedures, see Experimental Section. b For structures, see text.
Figure1. Compound 4a antagonizes the capacity of the cannabinoid agonists 7c (A) and 5a (B) to inhibit the forskolin-stimulate adenylylcyclase activity in CHO cells stably transfected with cDNA of human CB 2. Compounds 4a, 7c, and 5a

were added at the indicated concentrations; 100% represents the amount of cAMP in the absence of cannabinoids. Each point is the mean ± standard deviation of three experiments performed in triplicate.

the EC 50 for the CB 1 -mediated inhibition of cyclase was58.1 ± 6.2 nM, the inhibition of the CB 2 -mediatedcyclase was negligible: 20%inhibition at 10 μM. This result indicates that 4a could serve as a function alantagonist of CB 2 as it binds, but does not activate, CB 2 .Indeed, it reduces the CB 2 -mediated adenylylcyclase inhibitory activity of the potent agonists 5a (HU-210) 5,8b,20 and 7c (Figure 1). However while the adenylylcyclase inhibition by 7c is very strongly reduced by 4a, that of 5a is considerably less. This difference may well be due to the higher inhibitory potency of 5a ,compared to 7c. It should be pointed out that 4a, a major metabolite of CBN, in its differential activity between CB 1 and CB 2, resembles Δ 9-THC rather than CBN (see above): 12 both Δ 9 -THC and 4a bind to CB 1 and CB 2 and inhibit CB 1 -mediated cyclase in the nanomolarrange but cause only negligible inhibition of CB 2-mediated cyclase up to micromolar concentrations.The DMH homolog of 11-hydroxycannabinol (4b) is the most potent cannabinoid in the present series. It binds to CB 1 and CB 2 with K I values of 100± 50 and 200 ± 40 pM, respectively. Compound 4b also inhibits adenylylcyclase via both CB 1 and CB 2, at very low concentrations: 56.2 ± 4.2 and 207.5 ± 27.8 pM, respectively. Both the binding constants and EC 50 values for CB 1 and CB 2 are comparable to the values observed for HU-210 (5a). Cannabinol-11-oic acid (7a), 22 as well as its acetate (7b), were essentially inactive on binding to either CB 1 or CB 2, as well as in CB 1- or CB 2-mediated cyclase inhibition. However, the DMH homologs, compounds 7c, d, bind to both CB 1 and CB 2 and inhibit adenylyl-cyclase via the two receptors with high potency. The non acetylated derivative 7c binds with Ki values of 6.1 ± 1.1 and 4.8 ± 1.9 nM to CB 1 and CB 2, respectively, and inhibits cyclase CB 1-and CB 2-mediated activity with EC 50 values of 2.4 ± 0.2 and 5.4 ± 0.3 nM, respectively. These results were unexpected as THC-type 11-oic cannabinoid acids (including DMH ho-mologs) such as 6a, c have not been reported to cause psychotropic effects, 10,21,23 which are presumably mediated by CB 1.The acetate 7d is also active in all theseassays, although its activity is 4-7 times lower than the nonacetylated acid (see Table 1). This differencewas even larger (90 times) when the acetate 7d was compared to the nonacetylated acid 7c with regards to

binding to CB 1 on synaptosomal membranes. It is not clear whether the weaker activity of the acetate is due to its own lower intrinsic activity or to a certain amount of non acetylated 7c formed by hydrolysis during the assays. In view of these results (see Discussion), we looked again into the activity of the DMH homolog of Δ 8 -THC-7-oic acid (6a). 23 This compound, synthesized in ourlaboratory several years ago, was found then to reduce pawedema and leukocyte adhesion to culture dishesand was considered to be antiinflammatory. It did notcause catalepsy in mice at doses up to 1 mg/kg. 23 Wenow find that 6a binds to CB 1 with a rather low Ki of 32.3 ± 3.7 nM but is only a relatively weak inhibitor ofCB 1-mediated cyclase (EC 50 ± 927.0 ± 39.6 nM). Itbinds to the CB 2 receptor with a Ki of 170.5 ± 7.8 nM and inhibits CB 2-mediated cyclase (EC 50 ± 116.2 ± 74.7nM). This material thus affects signaling via CB 2 moreeffectively than via CB 1. The last series to be examined were cannabinol derivatives in which the pyran ring was modified; instead of the two methyl groups on ring B, a ketonewas introduced, thus forming a lactone. Compound 8a (with a pentyl side chain) 24 did not bind to either CB 1 or CB 2 and did not inhibit adenylylcyclase, while theDMH derivative 8b was highly potent, binding to both CB 1 and CB 2 with Ki values of 32.7 ± 4.9 and 17.3 ± 5.6 nM, respectively. It inhibited both CB 1 - and CB 2-mediated adenylylcyclase with EC 50 values of 9.6 ± 0.6 and 4.6 ± 0.3 nM, respectively.

Discussion

For reasons discussed in the Introduction, we assumed that CBN derivatives may show differential binding for CB 1 compared to CB 2 .The results obtained did not fulfill these expectations. Compounds binding poorly to CB 1 (such as 7a, 8a) had the same profile with CB 2, and this was the case also for the more potent compounds (such as 1b,4a,b,7c,d,8b). However, our results proved to be of interest in another direction: we found that binding to CB 2 (see compound 4a) does not necessarily imply a parallel level of inhibition of CB 2-mediated adenylylcyclase. As mentioned above, the major CBN metabolite, 11-hydroxy-CBN (4a), binds well to both CB 1 and CB 2 (Ki) 38 (7.2 and 26.6 (5.5 nM, respectively). However, while 4a inhibits adenylylcyclase strongly via CB 1 (EC 50) 58.1 (6.2 nM), its inhibition of adenylylcyclase via CB 2 is negligible (Table1). Further experiments (see Biological Results and Figure 1) indeed showed that 4a is a weak antagonist to CB 2. Hence, this CBN metabolite may represent a useful target for future research and possibly a tool in CB 2 investigations. As 4a is formed in vivo from CBN, its presence in the body may have physiological consequences associated with CB 2-promoted activities, possibly in the immune system. The replacement of the pentyl group at position 3 with the DMH group increased affinity to CB 1 as well as to CB 2 in all compounds tested. The dramatic increase of pharmacological activity associated with such a structural change was first noted about 50 years ago. 24c,d As indicated above, the DMH homolog of 11-hydroxy-CBN (4b) is the most active compound in the present series as regards binding to CB 1 and CB 2, as well as inhibition of adenylylcyclase (see Table 1). It may serve alongsideHU-210 (5a) as a potent tool in cannabinoid research.

Indeed, 4b shows binding values and cyclase inhibition levels very similar to those of HU-210 (5a) (see Table1).

Oxidation of the 11-CH 3 group in Δ 9 -THC (2a) or Δ 8-THC (12) to a carboxyl group forming THC-7-oic acids (13 or 6c, respectively) leads to inactivation, as seen inbehavioral assays. 10,21 Indeed, this route is the major inactivation pathway of THC metabolism. The DMH homolog of Δ 8-THC, 11-oic acid 6a, has also beenreported to lack THC-type activity, 23 although thereported test range was limited. Unexpectedly, we now find that although 6a binds to both CB 1 and CB 2 in the 30-170 nM range, the EC 50 of adenylylcyclase inhibi-tion via CB 1 is in the range of 927 ± 39.6 nM. The CB 2 -mediated adenylylcyclase is in the intermediate range(116.2 ± 74.7 nM), i.e., a certain separation betweenCB 1- and CB 2-mediated activation is noted. In theCBN-11-oic acid series, we observed a different profile:while in the pentyl series (7a,b), as expected, werecorded no binding or inhibition of cyclase, with DMH-CBN-11-oic acid (7c), we found potent binding to bothCB 1 and CB 2 and cyclase inhibition (see Table 1).Compounds 6a and 7c differ in the conformation of ringA: a planar aromatic ring in 7c versus a half-chair onein 6a . This conformational difference may represent the molecular basis for the different activities of the two cannabinoids.A comparison of the binding values of 6a with those of its CB 1- and CB 2

-mediated cyclase inhibition (see Table 1) may explain some of the previously reportedproperties of 6a. The relatively high levels of 6a needed to inhibit adenylylcyclase via CB 1 may explain the absence of catalepsy (within the limited dose range tested), in spite of its considerable binding potency,while the ca. 10 times lower levels needed to inhibit adenylyl cylcase via CB 2 may be the basis of its reported antiinflammatory activity. These observations mayserve to open new leads toward the development of antiinflammatory agents in which wider separations of activity can be achieved than these reported now. As mentioned above, all compounds presented inTable 1 were tested for binding both in transfected cellsand in rat brain synaptosomal preparations. While the general potency trend is comparable in the two assays, some individual differences are striking. Compounds 6b,7b, and 8a are inactive in both assays; 7a is inactivein transfected cells and poorly active in the membraneassay. The highly potent 1b,5a, and 7c have the same profile in both assays. However 11-hydroxy CBN-DMH (4b) has a Ki of 100.0 ± 50.0 pM in transfected cells and nearly 20 times higher Ki (i.e., lower potency) in brain membranes. In the compounds within the inter-mediate range, the Ki values differ widely: from about2 times (i.e., essentially equipotent) in 1a,2a, and 8b to about 10 times or morein 7d and 6a. The reasonsfor these differences are not clear, but they should be taken into account when binding data in the cannab- inoid series are compared.In summary, a comparison of the binding potency ofseveral cannabinol derivatives to CB 1 and CB 2 with

their capacity to inhibit adenylylcyclase has led to the discovery of a CBN metabolite (11-hydroxy-CBN, 4a) as a specific, though not potent, CB 2 antagonist and toa new very potent agonist for both CB 1 and CB 2 (11-hydroxy-CBN-DMH, 4b).

Experimental Section

Chemistry.

1 H NMR spectra were measured on a Varian VXR-300S spectrophotometer using TMS as the internal standard. All chemical shifts are reported in ppm. Specific rotations were detected with a Perkin-Elmer 141 polarimeter. Melting points (uncorrected) were determined on a Buchi 530 apparatus. Column chromatography was performed with ICN silica gel 60A. Organic solutions were dried over anhydrousmagnesium sulfate. Elemental analyses were obtained for all new compounds (or their acetates) and were (0.4% of the theoretical values. The analyses were performed at the Elemental Analysis Laboratory of the Hebrew University. Δ 9-Tetrahydrocannabinol-DMH (2c). Boron trifluorideetherate (5.5 mL) was added to cannabidiol-DMH (3b) 17 (5.7g, 15.4 mmol) in dry dichloromethane (150 mL) containing magnesium sulfate (1 g), under a nitrogen atmosphere. The reaction mixture was stirred at room temperature for 20 min. A saturated solution of sodium bicarbonate was added until the red color observed during the reaction faded. The reactionmixture was washed with water, separated, dried, and evapo-rated. The oil obtained was chromatographed on a silica gelcolumn. Δ 9-Tetrahydrocannabinol-DMH (2c) (3 g, 53%) waseluted with 4% ether in petroleum ether: 1 H NMR (CDCl 3) δ 6.38, 6.26 (s, 2H), 6.3 (s 1H), 3.16 (d, 1H, J) 11.1 Hz), 2.14(2H), 0.8-1.8 (m); IR (neat) 3300, 2950, 2850, 1620, 1570 cm-1 .Anal. (C 25 H 38 O 2) C,H. Δ 9- THC-DMH (2c) is not stable. At room temperature itrapidly becomes violet; on TLC after 0.5 h, numerous new spotsare observed. Δ 9- Tetrahydrocannabinol-DMH acetate(2d): 1 H NMR(CDCl 3 δ 6.67, 6.50 (s, 2H), 6.0 (s, 1H), 3.2 (d, 1H), 2.28 (s,3H), 2.14 (2H), 1.9-0.8 (m); IR (neat) 2900, 1760, 1620, 1560cm-1. Dehydrogenations of 2d,5b,6b,d, and 11b. The dehy-drogenations were carried out by heating each compound withsulfur at 238-240 °C, under a nitrogen atmosphere, for ca. 4h. Each mixture was extracted with ether and evaporated. The residue was chromatographed on a silica gel column using variable concentrations of ether in petroleum ether as eluent. Compound 2d led to cannabinol-DMH acetate (1c); compound 5b gave 4c; compound 6b gave 7d; compound 11b gave 8b .For yields and spectroscopic data, see below. Cannabinol-DMH acetate (1c): obtained in 24% yield, mp84-86 °C; 1 H NMR (CDCl 3) δ 7.8, 7.25, 7.13 (s, 3H), 6.85 (d,1H, J) = 2.1 Hz), 6.67 (d, 1H, J) 2.1 Hz), 2.36 (s, 3H), 2.32 (s,3H), 1.6-0.8 (m); IR (neat) 2930, 1780, 1620, 1560 cm - 1 . Anal.(C 27 H 36 O 3) C,H. 11-Hydroxycannabinol-DMH acetate(4c): obtained in44%yield from 5b; 1H NMR (CDCl 3) δ 8.00, 7.25 (s, 3H) 6.85(d, 1H, J) 1.5 Hz), 6.68 (d, 1H, J) 1.5 Hz), 5.10 (s, 2H), 2.34(s, 3H), 2.10 (s, 3H), 1.6-0.85 (m); IR (neat) 2930, 1780, 1740,1620, 1540 cm- 1. Cannabinol-11-oic acid acetate (7b): obtained in 22%yield from 6d , mp 149 -151 °C; 1 H NMR (CDCl 3) δ 8.81 (s,1H), 8.02 (d, 1H, J) 8.1 Hz), 7.38 (d, 1H, J) 8.4 Hz), 6.78,6.64 (s, 2H), 2.42 (s, 3H), 1.7 - 0.8 (m); IR (neat) 2960, 1760,1670, 1610, 1570 cm-1. Anal. (C 23 H 26 O 5) C, H. Cannabinol-11-oicacid-DMH acetate(7d): obtained in20% yield from 6b; 1 H NMR (CDCl 3) δ 8.8 (s, 1H), 8.02 (d, 1H, J) 8.1 Hz), 7.38 (d, 1H, J) 8.4 Hz), 6.86, 6.75 (s, 2H), 2.42(s, 3H), 1.7- 0.8 (m); IR (neat) 2960, 1780, 1680, 1620, 1570cm -1. Anal (C 27 H 34 O 5) C,H. Cannabinol-DMH (1b) : Hydrolysis of 1c in ethanolicsodium hydroxide solution gave compound 1b, mp 95-98 °C; 1 H NMR (CDCl 3) δ 8.11, 7.22, 7.08, 6.36, 6.4 (s, 5H), 2.33 (s,3H), 1.5-0.7 (m). 11-Hydroxycannabinol-DMH (4b): Compound 4c (526mg, 1.13 mmol) in dry ether (10 mL) was added to lithiumaluminum hydride (123 mg) in dry ether (8 mL). The mixturewas boiled under reflux for 2 h. The oil obtained after workup was chromatographed on a silica gel column (60 g). Elution with 20%ether in petroleum ether gave, after crystallization from pentane, compound 4b (340 mg, 78%): mp 128-130 C; 1 H NMR (CDCl 3) δ 8.45, 7.255, 7.253, 6.60, 6.40 (s, 5H), 4.75(s, 2H), 1.65-0.8 (m, 2H). Anal. (C 25 H 34 O 3) C,H. Cannabinol-11-oic acid-DMH (7c). Compound 7d (60mg) was disolved in 0.6 mL of ethanol. A solution of 60 mg of sodium hydroxide in 0.4 mL of water was added undernitrogen. The solution was stirred at room temperature for 30 minutes. The solution was acidified with 10%HCl (5 mL); the mixture was extracted with ether, dried with MgSO 4, and evaporated. The material obtained was separated on apreparative TLC plate (elution with ether: petroleum ether,7:3), leading to 41 mg of 7c, 76%yield: 1 H NMR 9.2 (s, 1H),8.0 (d, 1H), 7.38 (d, 1H), 6.61 (s, 1H), 6.45 (s, 1H), 1.7- 0.8(m). Cannabinol-11-oicacid(7a):7b was hydrolyzed in etha-nolic sodium hydroxide solution as described for compound 7c to yield 7a, 82%yield; 1 H NMR δ 9.2 (s, 1H), 8.0 (d, 1H), 7.38(d, 1H), 6.62 (s, 1H), 6.47 (s, 1H), 1.8-0.8 (m). Compound11b. A solution of 3-DMH-resorcinol (9b) (3.9g, 16.5 mmol), ethyl 4-methyl-2-oxocyclohexane carboxylate (10) (4 g, 21.7 mmol), and POCl 3 (3.06 mL) in dry benzene (15 mL)was boiled under reflux, under a nitrogen atmosphere, for 3h. The solution was washed with NaHCO 3 followed by water. After drying and evaporation the oil was chromatographed ona silica gel column. Compound 11b 24c (40%) was eluted with 10% ether in petroleum ether: mp 158-160 °C (from pentane); 1 H NMR (CDCl 3) δ 6.83, 6.63 (s, 2H), 3.4 (dd, 2H), 2.8 (dd,2H), 2.7-0.8 (m); IR (Nujol) 1680, 1610sh, 1580 cm-1; MS M + 356. Compound 8b: dehydrogenation of 11b with sulfur asdescribed above gave compound 8b (72%), mp 184-185 °C(from pentane); 1 H NMR (CDCl 3) δ 8.82 (s, 1H), 8.3 (d, 1H),7.38 (d, 1H), 6.94, 6.72 (s, 2H), 2.54 (s,

3H), 1.6-0.8 (m, 19H).Anal. (C 23 H 28 O 3) C,H. Receptor Binding Assay. a. Binding to Synaptosomal Membranes. Synaptosomal brain preparations were made from whole rat brain as described previously. 26,27 Binding of [3 H] HU- 243 (50.4 Ci/mmol) was assayed in triplicate asdescribed. 26,27 In brief, each reaction mixture of 1 mL insiliconized Eppendorf tubes contained 2.4- 3.8 µ g of synapto somal membrane protein, 28-48 fmol of [3 H] HU-243, and various concentrations of competing unlabeled cannabinoids. Tubes were incubated at 30 °C for 90 min and centrifuged at13 000 rpm, and the tips of the tubes containing the pelleted membranes were cut and counted for their radioactivity. b. BindingtoTransfectedCOS-7Cells. Two days after transfection (with 5 µ g/100 mm of dish plasmids encoding CB 1 or CB 2) the cells were washed with phosphate-buffered saline, scraped, pelleted, and stored at-80 °C. Cell pellets were homogenized in binding buffer (50 mM Tris-HCl, 5 mM MgCl 2, and 2.5 mM EDTA, pH 7.4), and 50 µ g protein aliquots were assayed for binding of [3 H] HU-243 as described above, except that the final concentration of [3 H] HU-243 was 300 pM. Formore detailed information see refs 11 and 12.Specific binding was defined as the difference between the amount of radioactivity bound to the pelleted membranes in the absence and presence of 50 nM unlabeled HU- 243 (for a)or 1 µM unlabeled 5a (HU-210) (for b) and was typically 70-80% of the total bound. The Ki values for the various cannabinoids and related compounds were calculated from the competition data according to the formula: Ki) IC 50 /1 + ([3 H]-HU-243/ K d). 29 The K d values for HU-243 binding were 45 26 and 61 pM 11 for CB 1 and CB 2 , respectively. Cell Cultures. Cells were cultured in Dulbecco’s modified Eagle's medium supplemented with 8%fetal calf serum, 2 mM glutamine, nonessential amino acids, 100 units/mL penicillin, and 100 µ g/mL streptomycin in a humidified atmosphere consisting of 5%CO 2 and 95%air at 37 °C. CHO cells stably transfected with the cDNA of human CB 2 receptor 9 were described earlier. 11 COS-7 cells in 100 mm dishes were transiently transfected 12 with plasmids encoding rat CB 125 or human CB 29 (5 µ g each) and, when indicated, with 2 µ g of the plasmid containing the cDNA of adenylylcyclase type V. 12 Twenty-four hours later, the cells were trypsinized and cultured in 24-well plates. After an additional 24 h, the cells

were assayed for adenylylcyclase activity. Transfection efficiency, determined by transfection with the cDNA for ?-ga-lactosidase, was 40-80%. Adenylylcyclase Assay. The assay were performed as described. 11,12,27 Cells cultured in 24-well plates were incu-bated for 3 h with 0.25 mL/well fresh growth medium contain-ing 5µCi/mL [3 H] adenine. This medium was replaced with Dulbecco's modified Eagle’s medium containing 20 mM Hepes (pH 7.4), 1 mg/mL fatty acid-free bovine serum albumin, 0.5mM 1 methyl-3-isobutylxanthine, and 0.5 mM RO-20-1724.Cannabinoids and forskolin (1 µM) were added, and the cells were incubated at 37 °C for 10 min. The reaction was terminated with 1 mL of 2.5%perchloric acid containing 0.1mM unlabeled cAMP. Aliquots of 0.9 mL were neutralized with 100 µL of 3.8 M KOH and 0.16 M K 2 CO 3 and applied toa two-step column separation procedure. 12,27 The [3 H] cAMP was eluted into scintillation vials and counted. Background levels (cAMP accumulation in the absence of forskolin) were subtracted from all values and represented less than 10%offorskolin stimulated cAMP accumulation. Statistical Analysis. Data were analyzed using the Stu dent’s t-test. Inhibition curves were generated with the Sigma Plot 4.11 program, and the EC 50 values were determined using an equation from the ALLFIT program. 30

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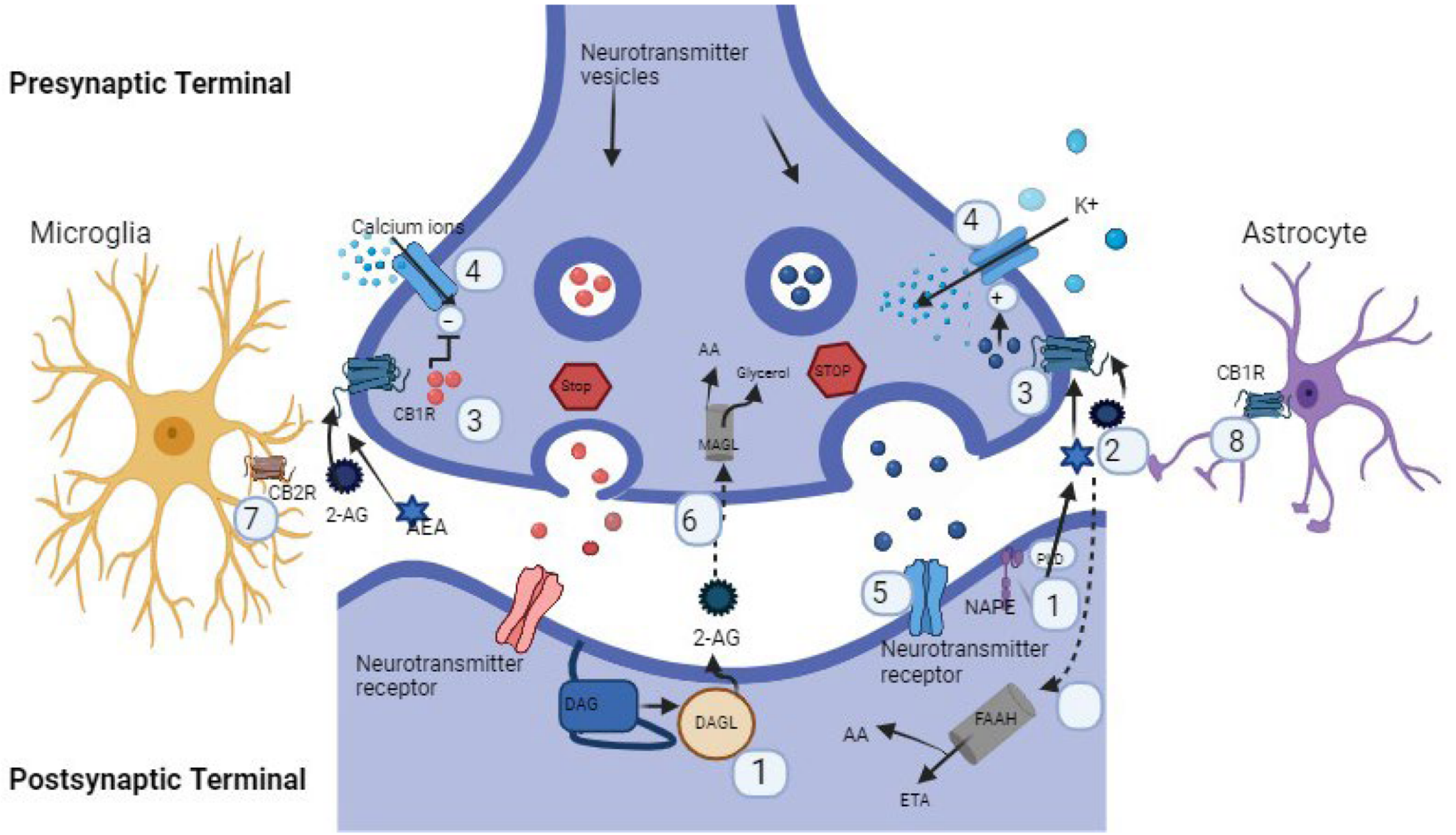

Modulation Of The Endo-Cannabinoid System: Therapeutic Potential Against Cocaine Dependence

Gianluigi Tanda

Abstract

Dependence on cocaine is still a main unresolved medical and social concern, and in spite of research efforts, no pharmacological therapy against cocaine dependence is yet available. Re- cent studies have shown that the endocannabinoid system participates in specific stages and aspects of drug dependence in general, and some of this evidence suggests an involvement of the cannabinoid system in cocaine effects. For example, cocaine administration has been shown to alter brain endocannabinoid levels, and the endocannabinoid system has been in- volved in long-term modifications of brain processes that might play a role in neuro/behavioral effects of psychostimulant drugs like cocaine. Human studies show that marijuana dependence is frequently associated with cocaine dependence, and that the cannabinoid receptor CNR1 gene polymorphism might be related to cocaine addiction. This article will review the main papers in the field showing how a modulation of different components of the cannabinoid sys- tem might interact with some of the neurobiological/behavioral effects of cocaine related to its reinforcing effects, evaluated in preclinical models or in clinical settings. The goal of this review will be to provide insights into the complex picture of cocaine abuse and addiction, and to extrapolate from such endocannabinoid-cocaine interactions useful information to test the therapeutic potential of cannabinoid ligands and endocannabinoid-level enhancers against co- caine dependence for future preclinical/clinical trials.

Keywords: cannabinoids, cocaine, addiction, behavior

1. INTRODUCTION

A number of studies suggest that the cannabinoid system interacts with cocaine's effects at dif- ferent levels in the brain. For example, both CB1 agonists and cocaine selectively increase dopamine neurotransmission in the nucleus accumbens shell (1, 2, 3, 4), a brain area believed to play a pivotal role in the addictive effects of drugs (5). It is also interesting to note that co- caine administration results in increased levels of anandamide, one of the endogenous ligands for cannabinoid receptors in the brain (6, 7), and that blockade of CB1 cannabinoid receptors attenuates a dopamine signal triggered by cocaine in the NAc (8).

The cannabinoid system has also been implicated in brain processes related to potentiation or depression of the strength of synaptic signals (9, 10, 11, 12). These so-called processes of long term potentiation and long-term depression activated by cannabinoid neurotransmission might play a significant role in those neurobiological adaptations which are believed to take an im- portant part in the road leading from drug use to drug abuse and addiction (13, 14, 15). More- over, cannabinoid receptors are located in brain areas that have been assigned a key role in brain functions linked to the development of drug-dependence (16). Plant-derived and endoge- nous cannabinoid ligands have been suggested to affect neurotransmitter transporter functions (17, 18, 19, 20, 21, 22), which are also pharmacological targets for cocaine (23, 24). Finally, it is interesting to note that cocaine dependence is often diagnosed in the presence of other drug dependencies (25), and marijuana dependence is second only to alcohol dependence as the most frequent comorbid dependence in cocaine addicts (25, 26).

1.1 Overview on cocaine abuse and addiction

Cocaine abuse and addiction are widespread throughout the world, and these phenomena are driven by the ability of this drug to produce reinforcing effects by interacting with specific sites in the central nervous system (23). Cocaine binds to specific transport sites on the cell mem- branes of neurons that release dopamine, serotonin or noradrenaline. These transport sites have the physiological function of

removing neurotransmitters from the extracellular space after their release from nerve terminals (24). Binding of cocaine to the neurotransmitters' transport sites results in blockade of the transport, increased availability of neurotransmitters for binding to their respective receptors, and ultimately increased neurotransmission (23, 27).

Although cocaine also binds and blocks the transporters for serotonin and noradrenaline (24), blockade of the dopamine transporter (DAT) appears to be the pharmacological target of co- caine mainly related to its reinforcing and other behavioral effects (23, 27). Thus, increased availability of dopamine for dopamine receptors is believed to mediate most of the reinforcing actions of cocaine, leading to its abuse liability (1, 3, 23, 27). However, cocaine abuse and ad- diction are processes that go well beyond the pharmacological effects of cocaine on dopamine neurotransmission, so that many other factors, genetic and environmental for example, have been suggested to play a role in the complex of actions that lead from cocaine use to its abuse and addiction (28, 29, 30, 31).

There is no specific pharmacological therapy available against cocaine's addictive effects, but researchers in the last decades have made progresses in the understanding of the neurobiology underlying cocaine's actions (29, 30). Today, different aspects of cocaine abuse and addiction in humans can be modeled and studied in experimental animals. These animal models have provided valuable results leading to a better understanding of the different factors contributing to cocaine effects (32). Moreover, the models have been invaluable in the study of newly syn- thesized compounds acting over different neuronal systems that might become candidates for development as cocaine abuse medication (29, 30).

1.2 Overview on the brain endocannabinoid system

The important discoveries in the last two decades in the cannabinoid field have provided useful information about the physiological functions of the endocannabinoid system in the brain and periphery, and the involvement of this system in the etiology and therapy of many human dis- eases (33, 34, 35). During the last decade researchers have provided evidence for the existence of at least two cannabinoid receptors, CB1 and CB2 (36, 37, 38, 39) and we have now avail- able different selective synthetic agonists and antagonists for both CB1 and CB2 receptors (40, 41), other than classic, plant-derived cannabinoid agonists like delta-9-THC, the active ingre- dient in marijuana. Although the existence of novel cannabinoid receptors has been suggested in different studies (42, 43, 44), definitive proof of the existence of these cannabinoid receptors has yet to be shown. Several different endogenous ligands for CB1 and CB2 receptors have been found, such as anandamide, 2AG, noladin-ether (45, 46, 47, 48). At variance with classical neurotransmitters, these endocannabinoids are not stored in vesicles in nerve endings, but are released "on de- mand" by specific changes occurring in neurons which promote the function of specific en- zymes that produce anandamide or 2-AG from membrane-constituent lipids (49, 50, 51, 52). We now have evidence that endogenous cannabinoids released "on demand" can function as retrograde messengers in the synapse (53). Thus, activation of cannabinoid receptors might change the electric equilibrium of the post-synaptic membranes with important modifications for cell-depolarization, neuronal firing, and release of classic neurotransmitters, which ulti- mately results in a modification of brain neurotransmission (10, 11).

Some of the neurobiological effects of endocannabinoids have been demonstrated to be long- lasting, leading to the so-called processes of long-term potentiation and long-term depression of the synaptic transmission strength (11, 12). It is interesting to note that these long-term ef- fects are in spite of the very brief half life of endogenous cannabinoid ligands in the extracellu- lar space (49, 54, 55).

Anandamide and 2-AG are taken-up into the cell by a not completely defined and still debated mechanism/s: passive diffusion or facilitated transport across the plasma membrane (55, 56, 57, 58, 59). Anandamide is then metabolized into the cell by an intracellular enzyme, the fatty acid amide hydrolase (FAAH) (60, 61), the blockade of which has been shown to prolong the behavioral/neurobiological effects of anandamide (4, 61, 62, 63). In contrast, 2-AG is metabo- lized through the mono glyceril lipase enzyme (54). In the last few years several pharmacologi- cal tools have been discovered that alter the physiological equilibrium of the endocannabinoid system by acting on the metabolic pathways of the endogenous ligands. The result of this mod- ulation is that blockade of endocannabinoid metabolism increases levels of endocannabinoids in specific brain areas in which there is a preexisting cannabinoid tone or where endogenous cannabinoids are released on demand triggered by specific neuronal signals (34, 61). These highly-specific effects contrast with the effects of exogenously administered cannabinoid ago- nists, which diffuse widely throughout the brain leading to a generalized and contemporaneous activation of multiple brain areas.

It is believed that in humans, cannabinoid receptor agonists, like delta-9-THC, activate cannabinoid CB1 receptors, producing reinforcing effects which are the basis for marijuana abuse (2, 64). CB1 receptors are also involved in other brain mechanisms and functions associ- ated with pathologies in which a cannabinoid intervention might have a therapeutic value (33, 34, 65, 66). Unfortunately, there is no cannabinoid agonist drug available yet that can selective- ly produce therapeutic effects without having undesired psychotropic, reinforcement-related effects. However, pharmacological tools are now available that can modulate the activity of endogenous cannabinoid ligands in selected brain areas, thereby having therapeutic value (34) without the harmful reinforcing effects produced by delta-9-THC-like drugs with the wide- spread action. Indeed, interesting actions of such cannabinoid modulators have been already described in preclinical models of anxiety (61, 67) and depression (68), and preclinical tests so far have also shown negative results in procedures involving models of drug abuse (4, 68, 69).

2. Effects of cannabinoid drugs or endocannabinoid modulators in preclinical models of cocaine's behavioral and reinforcing effects

2.1 Cannabinoid interactions with cocaine-induced stimulation of motor activity and behavioral sensitization

2.1.1 Effects after acute administration of cocaine Psychostimulants, like cocaine, have the abil- ity to influence neuronal activity, which results in stimulation of behavioral activity. Stimula- tion of locomotor activity induced by cocaine and other psychostimulants has been extensively studied (see, for example citations 70, and 71). Administration of psychostimulants in rodents induces a dose-dependent increase in behavioral activation, which depends on the activation of selected brain striatal areas (72, 73, 74, 75, 76), and that is accompanied by stimulation of DA neurotransmission (3, 77, 78, 79). Although this behavior is not a direct correlate of the rein- forcing effects of cocaine, the fact that it can be obtained by injecting cocaine in the NAc (72, 76, 77), and that NAc DA transmission is involved in different aspects of drug abuse and ad- diction (5, 80, 81), makes monitoring of locomotor activation an easy preclinical target to test drugs

that can interfere with the ability of cocaine to stimulate DA neurotransmission and po- tentially attenuate cocaine-induced reinforcement (82).

Docannabinoidsaltertheacuteeffectsofcocaineonstimulationofmotoractivities? Pryor and colleagues, 1978 (83), described the interaction between cocaine and delta-9-THC on mo- tor activity, measured as photo-beam interruptions in rats, as one of mutual antagonism depen- dent on the combination of doses tested. The antagonism was attenuated but still evident after sub-chronic administration of delta-9-THC. In agreement with these data, Ferrari et al., 1999 (84), showed that HU210, a synthetic agonist for CB1 receptors, dose dependently reduced the locomotor activity induced by cocaine, 15 mg/kg i.p. in rats. However, sub-chronic administra- tion of HU210 was not able to modify cocaine-induced locomotion at variance with the previ- ous report. It should be noted that a cannabinoid antagonist was not available in 1978, and that Ferrari et al., 1999 (84), did not test whether a CB1 receptor antagonist could interfere with HU210 actions on cocaine effects. Because of the lack of direct pharmacological involvement of CB1 receptors in cocaine effects, Przegalinski et al., 2005 (85), tried to address this issue in a more recent report. In that study the authors showed that the CB1 receptor agonist WIN 55,212-2, but not its less active enantiomer WIN 55,212-3, dose dependently (3–6 mg/kg i.p.) reduced cocaine-induced hyper-locomotion, without affecting behavior when administered alone. Rimonabant (SR 141716), a selective antagonist of CB1 receptors (86), did not reverse the effects of WIN 55,212-2 on cocaine-stimulated locomotion, suggesting a non-CB1 mediat- ed mechanism of action for WIN 55,212-2. Moreover, the effect was not attributable to unspe- cific interactions of the highly lipophilic cannabinoid agonist WIN 55,212-2 with hydrophobic sites of proteins or other cell's membrane lipid constituents, since the less active enantiomer did not show any significant effect on cocaine-induced hyper-locomotion (85). Further, the au- thors showed that the effects of WIN 55,212-2 resembled those of a serotonin 5HT3 antagonist, based on other previously published evidence (87, 88), thus indicating the possibility of an in- volvement of 5HT3 receptors in the action of cannabinoid agonists on cocaine-induced hyper- locomotion. However, other than a resemblance of effects, there was no direct pharmacological evidence for 5HT3-receptor involvement in the actions of WIN 55,212-2 on cocaine-induce hyper-locomotion (85). It should also be noted that in the report by Przegalinski et al., 2005 (85), the locomotor stimulant effects of cocaine were reduced to a certain extent, though not significantly, by Rimonabant itself, while in a recent report (8) Rimonabant significantly atten- uated cocaine-induced hyperactivity, thus suggesting the possibility that both agonist and an- tagonists of CB1 receptors can negatively modulate the locomotor stimulant effects of cocaine. Also, Rimonabant antagonized cocaine-induced hyper-locomotion in Gerbils habituated to the test cage, but not in non-habituated animals (89). The authors explained their results in terms of a cannabinoid involvement as a permissive element required in the ability of cocaine to override novelty-stimulus satiation in habituated Gerbils. However, lack of effects of Rimona- bant on acute cocaine-induced locomotor stimulation has also been reported in habituated mice and rats (90, 91).

Taken together these experiments suggest that an endocannabinoid tone might not be necessary to mediate the acute motor stimulant effects of cocaine in rodents. However, depending on dose, both cannabinoid CB1 receptor agonists and antagonists might modulate this behavioral stimulation induced by cocaine. Moreover, as shown by Cheer et al., 2007 (8), attenuation of cocaine-induced locomotor activity by a CB1 receptor antagonist has a neurochemical corre- late. In that study, Rimonabant pretreatment attenuated dopamine neurotransmission, as mea- sured by "fast-scan-cyclic voltammetry", suggesting that a cannabinoid tone might be involved in both neurochemical and behavioral processes triggered by cocaine (8). 2.1.2. Effects in cocaine-sensitized animals Psychostimulant sensitization is a phenomenon that has been extensively described in rodents. It appears as an increased effectiveness of the psy- chostimulant drugs to produce behavioral activation in animals previously exposed to single (92) or repeated (93) administration of the same drug. Cross sensitization between different drugs has also been described (94, 95).

It has been suggested that sensitization induced by drugs of abuse may be due to neuronal adaptations that play a role in the development of addictive behaviors. Behavioral sensitization is easily demonstrated in animals, and it has been hypothesized that this adaptation is involved in the transition from drug use to abuse and addiction (see citations 14, and 15, for review). Thus, behavioral sensitization has been the target of different studies searching for drugs able to impair or reduce the effects of this adaptation induced by sub-chronic/chronic administration of cocaine.

Do cannabinoids play a role in the behavioral sensitization induced by cocaine? There are only a few reports about cannabinoid interactions with development or expression of cocaine- induced behavioral sensitization. Sensitization and cross sensitization have both been reported for cannabinoid agonists and some drugs abused by humans including psychostimulant drugs (94, 95, 96, 97, 98, 99, 100). However, failure to develop cross sensitization in rats chronically treated with a cannabinoid agonist, CP 55,940, and challenged with cocaine has also been re- ported (101). In the same study, CP 55,940 failed to enhance the sensitivity to cocaine when the drugs were concurrently administered during the development phase. Also, the develop- ment of cocaine sensitization did not appear to be under control of CB1 receptors in rodents in another report (90). In this latter study, mice were treated with 20 mg/kg cocaine for 11 days in their home cages. Three days after the last cocaine injection they habituated to an open field, and then tested with cocaine (10 mg/kg). The authors reported a first set of experiments in which Rimonabant 1 mg/kg administered 30 min before the daily cocaine injection significant- ly reduced the locomotor sensitization induced by cocaine. Such effects, however, were not replicated in a subsequent set of experiments with increasing doses of cocaine (90).

In genetically modified CB1 receptor knock out mice (102), cocaine (10 mg/kg twice daily for 15 days) induced a behavioral sensitization, tested after one week withdrawal in an open field, which was indistinguishable from that induced in wild-type littermate control mice. These findings once more suggest a lack of involvement of the endocannabinoid system in the devel- opment of behavioral sensitization induced by cocaine. On the other hand, the expression of cocaine sensitization has been demonstrated to be impaired by cannabinoid CB1 receptor blockade, as shown by administration of the selective CB1 receptor antagonist Rimonabant together with cocaine five days after withdrawal from cocaine (103). Although this interaction needs to be further investigated, results from the cited reports suggest that even if activation of cannabinoid neurotransmission through CB1 receptors might not be required for development of cocaine-induced sensitization, an endogenous cannabinoid tone might be essential for its expression.

Thus, cannabinoid antagonists might decrease the impact of a “sensitized” response to drug stimuli in subjects during abstinence. This prediction matches the results obtained by DeVries et al., 2001 (104), with administration of the cannabinoid receptor antagonist Rimonabant in cocaine abstinent rats during self-administration reinstatement tests (see below the section: “Effects of cannabinoids on animal models of extinction and reinstatement of cocaine-seeking behavior”).

2.2 Cannabinoid interactions with cocaine-induced place conditioning

The place conditioning procedure is a model of drug reinforcement and is based on an individ- ual’s behavioral choice after a certain number of pairings of drug with one environment and placebo with another. This procedure, though simple, can give an indication of the pleasurable (place preference) or aversive (place avoidance) feelings/perceptions induced by drugs during the conditioning procedure. The place-conditioning allows the study of both development and expression of aversion or preference for a place associated with a certain drug dose (see cita- tions 105, and 106 for review), with the final test performed with animals in a drug-free state. So, the ability of drugs to interfere or impair the effects of the conditioning-drug could be re- lated to a potential therapeutic value, in terms of ability to reduce or block the pleasurable feel- ings induced by the conditioning drug, or to attenuate the strength of the association between the conditioning drug and the environmental stimuli.

It has been repeatedly shown that cocaine, just like many other drugs abused by humans, sup- ports place preference at certain doses (106). On the other hand, results from place condition- ing studies with cannabinoid agonists and antagonists have been inconsistent (see citation 107 for review), and both preference and aversion induced by cannabinoid CB1 receptor agonists and antagonists have been reported under a variety of different experimental conditions and dose ranges (108, 109, 110, 111, 112, 113).

The involvement of the cannabinoid system in processes related to positive reinforcement in- duced by cocaine in the place conditioning procedure was evaluated by Chaperon et al., 1998 (91), in rats. The authors showed clear evidence that blockade of CB1 receptors by administration of the selective cannabinoid CB1 receptor antagonist Rimonabant impaired the acquisition of cocaine-conditioned place preference, suggesting that the development of such conditioning was under control of a cannabinoid tone in the brain. In the same paper the authors also showed that blockade of CB1 receptors did not impair the expression of cocaine-conditioned place preference when the antagonist of CB1 receptors, Rimonabant, was administered only once, 30 min before the final test, instead of 30 min before each cocaine injection during the conditioning sessions (91). At variance with this report, results from a study by Martin et al., 2000 (102), showed that development and expression of cocaine-conditioned place preference can be established in genetically modified CB1 receptor knock-out mice. These latter results suggest no involvement of the endogenous cannabinoid systems in brain processes related to the motivational effects induced by cocaine in this procedure in genetically modified mice (102). These contradictory findings (91, 102) might be explained on the basis of species differ- ences, or developmental adaptations in the regulatory processes involved in the mediation of cocaine reinforcing effects in mice genetically deficient in CB1 cannabinoid receptors. It should be noted that Rimonabant might have inverse agonist actions on CB1 receptors (114, 115, 116), or might possess actions over pharmacological targets other than on CB1 receptors, for example on the putative CB3 receptor (42, 65). Martin and colleagues (102) did not cite the article by Chaperon et al., 1998 (91), so we do not have their comments on this issue. We also do not have information about the effects of Rimonabant administration on development and expression of cocaine-induced place preference in wild-type mice, an experiment that would allow a direct comparison with data obtained in CB1 KO mice (102).

2.3 Effects of cannabinoids on the discriminative stimulus effects of cocaine

The drug discrimination procedure has been extensively used in animal and human subjects to evaluate the specific perceptions/feelings produced by drug administration (117). It is likely that these subjective effects play an important role in the reinforcing effects of many drugs abused by humans. Indeed, many of these drugs produce euphoric or pleasurable sensations that are believed to be factors contributing to the abuse of substances (117).

The discriminative stimulus properties of cocaine have been evaluated in many differents stud- ies under different conditions and in different species, including human subjects (117, 118). However, there is a very limited set of data available in which the influence of the cannabinoid system has been evaluated in animals trained to discriminate the subjective effects of cocaine from those of placebo. Delta-9-THC did not substitute for cocaine in pigeons trained to dis- criminate cocaine from placebo, or cocaine from morphine, suggesting that the discriminative stimulus of the cannabinoid agonist and that of cocaine do not overlap (119, 120). In a more recent report (103) the cannabinoid receptor antagonist Rimonabant did not substitute for co- caine, and did not change significantly the discriminative stimulus effects of cocaine in rats. These limited findings indicate that the endogenous cannabinoid tone mediated by CB1 recep- tors does not play a role in the expression of the discriminative stimulus of cocaine. However, more detailed studies with a complete range of cannabinoid drugs (agonist, antagonist, and modulators) and doses are needed to further elucidate the role of the cannabinoid system in the subjective effects of cocaine.

2.4 Cannabinoid modulation of the effects of cocaine on thresholds for Intra Cranial Self Stimulation (ICSS)

This procedure is based on studies by Olds and Milner (121), in which rats have the opportuni- ty to learn to repeatedly press a lever in order to obtain an electrical stimulation of the medial forebrain bundle, a component of reward circuits in the brain (122). It has been repeatedly shown that most drugs of abuse are able to lower ICSS thresholds in a dose-dependent fashion (122, 123). This effect has been used as an indirect measure of their ability to produce “re- ward” through brain processes linked to the medial forebrain bundle. Thus, substances able to reduce or block the ability of drugs of abuse to lower the ICSS threshold may have therapeutic potential as treatments for drug abuse. While the effects of cocaine in this procedure are con- sistent with its reinforcing effects in animal and human subjects, inconsistent results have been obtained with administration of cannabinoid drugs (124, 125; see also citations 106, and 126, for review). There is only limited information available for combinations of cocaine and cannabinoids studied with the intracranial self stimulation procedure. In a study by Pradhan et al., 1978 (127), co-administration of delta-9-THC in rats resulted in an antagonism of the ef- fects of cocaine, while in experiments in which delta-9-THC was administered 80 min before cocaine, the effects of cocaine were unaffected by the cannabinoid

agonist. In agreement with this study, in a more recent report (128) administration of the synthetic cannabinoid agonist WIN 55,212-2 reduced the effects of cocaine on ICSS thresholds. In the same study, adminis- tration of the CB1 receptor antagonist, Rimonabant, antagonized the effects of WIN 55-212,2 on cocaine. Also, Rimonabant administration did not affect cocaine actions on this procedure, though only very low doses of the CB1 antagonist were tested (128).

These above cited reports suggest that cannabinoid agonists might have some potential activity against cocaine reinforcing effects in rodents, however, the mechanism/s for these interactions are not clear. Drugs of abuse and intra cranial self stimulation produce reinforcing effects through the same reward circuitry (122), and also increase dopamine neurotransmission in a dopaminergic reward-related area of such circuitry (1, 2, 3, 78, 129, 130) that has been related to their reinforcing effects (5, 79, 80). Thus, it is not apparently clear why cocaine and cannabi- noid CB1 receptor agonists, which share the ability to stimulate the brain reward circuitry, may have different and antagonistic effects in this procedure. However, the fact that in rodents cannabinoid agonists do not consistently produce reinforcing effects, but do so only under cer- tain experimental conditions (108, 124, 131, 132, 133, 134; see also 107, 126, and 135), sug- gests a cautious approach to the outcome of experiments involving reinforcing effects of cannabinoids in these species as compared to the well known consistent reinforcing effects of marijuana in human subjects.

2.5 Effects of cannabinoids on cocaine-maintained self-administration behavior

Although the most direct outcome of this procedure is to predict reinforcing effects and abuse liability in humans for drugs that maintain self-administration behavior in experimental ani- mals, many other aspects of this behavioral procedure can contribute to a better understanding of the behavioral effects of drugs and drug-related cues, their related neurobiology and poten- tial therapeutic interventions (136, 137).

As a drug that is widely abused, cocaine consistently maintains intravenous self-administration behavior across a range of doses in a variety of different species (see, for example, citations 138, and 139). Although the reinforcing effects obtained by administration of plant derived cannabinoids in humans were described in books thousands of years ago, cannabinoid agonists do not consistently maintain self-administration behavior in experimental animals (see cita- tions 107, 126, and 135 for review).

Preclinical studies have shown that pharmacological alterations of the endocannabinoid recep- tor system might interfere with the ability of cocaine to maintain self-administration behavior. In Long Evans rats, administration of the cannabinoid agonist WIN 55-212,2 reduced the aver- age number of intravenous self-injections of different doses of cocaine under a fixed-ratio 1 schedule, suggesting a potentiation of cocaine effects (140). In the same report, the selective antagonist of CB1 receptors Rimonabant antagonized the effects of WIN 55-212,2 on cocaine self-administration behavior. Rimonabant, however, did not affect the behavior maintained by cocaine when administered alone (140). In agreement with this report, Rimonabant pretreat- ment did not show any effect on cocaine self-administration under fixed-ratio schedules of be- havior in squirrel monkeys (63), an effect that has been recently replicated and confirmed (141, 142). In addition, Rimonabant did not affect the number of cocaine self-injections in Wistar rats studied under a fixed-ratio 5 schedule of drug delivery (103, 104). In agreement with the previous experiments in rats and primates, pre-treating C57Bl/6J mice with Rimonabant before single one-day sessions of cocaine self-administration affected neither the number of nose- pokes maintained nor the total cocaine intake during the 30-min session (90). In contrast the effects of genetic deletion of CB1 receptors have not been entirely consistent, and can differ with the effects of administering the pharmacological antagonist of CB1 receptors, Rimona- bant. For example, using the single one-day cocaine self-administration procedure mutant cannabinoid CB1 receptor knock-out mice failed to self-administer morphine, but were still able to self-administer cocaine, suggesting that the endocannabinoid system was a substrate for mediating the reinforcing effects of opioids but not those of cocaine (143). At variance with these findings, a more recent report, using the same mutant cannabinoid CB1 receptor knock- out mice as subjects, showed a reduced number of mice reaching the acquisition criteria, which was also obtained within a lager number of sessions, and a significantly reduced reinforcing efficacy of cocaine (measured as the effort required to obtain a cocaine infusion) as compared to wild-type littermate control mice (144). The authors explain their results, contrasting with those of Cossu et al., 2001 (143), in terms of different experimental conditions; freely moving mice chronically receiving cocaine self-infusions in daily sessions (144) versus mice with re- strained mobility receiving cocaine during a single session (143).

Soria and colleagues (144) confirmed their data in mutant CB1 receptor knock-out mice study- ing the effects of pretreatments with the antagonist of CB1 receptors Rimonabant in wild-type mice on cocaine self-administration under a progressive-ratio schedule. They found that Ri- monabant dose-dependently reduced the breaking point achieved by wild-type mice under the progressive-ratio schedule of cocaine self-administration, suggesting that also in normal con- trol mice the endocannabinoid system was a substrate for the reinforcing effects of cocaine (144). It is interesting to note that in this report the authors excluded any involvement of learn- ing/memory processes in the effects of cocaine in mice lacking the CB1 receptors because the same mice successfully acquired an operant response maintained by natural reinforcers (food and water). Also, the reduced efficacy of cocaine as a reinforcer was not the result of a dimin- ished ability of cocaine to stimulate dopamine neurotransmission, measured by intra-cerebral microdialysis in limbic areas of mutant mice as compared to their littermate controls (144). These latter results point toward a more complicated and central involvement of the endo- cannabinoid system in the addictive effects of cocaine.

As neither deletion nor blockade of CB1 receptors impairs cocaine’s ability to stimulate DA neurotransmission, it seems that the effects of modulation of the endocannabinoid system on cocaine self administration may be indirect. In a recent report using Sprague Dawley rats, prior exposure to delta-9-THC did not affect the acquisition and subsequent rates of cocaine self- administration under a fixed-ratio 1 schedule of injection, but did reduce the number of “co- caine-seeking” responses under a progressive-ratio schedule (145). Thus the sensitivity to delta-9-THC exposure may vary with behavioral procedure. Indeed, it has been already report- ed that changes in reinforcing efficacy of cocaine are hard to detect using fixed-ratio schedules of cocaine self-administration (146). In the report by Panlilio and colleagues (145), delta-9-THC pre-exposure also decreased both the number of entries in the center-zone of an open- field arena, and the time spent in the lighted compartment of the dark-light box (two different predictors of an anxiety response) in cocaine-treated rats. The

authors suggested that the re- duced effectiveness of cocaine as a reinforcer after delta-9-THC pre-exposure may be because the previous exposure to the cannabinoid agonist enhanced the aversive/anxiogenic effects of cocaine (145).

The contrasting results between species and schedules of drug delivery together suggest that the endocannabinoid system might impair other brain processes related to cocaine self admin- istration which might in turn reduce the ability to detect the reinforcing effects of cocaine. Cer- tainly, a better understanding and clinical prediction of the influence of the endocannabinoid system on the reinforcing effects of cocaine will come from studies of the effects of cannabi- noid agonists and antagonists under a variety of procedures (including progressive-ratio sched- ules) and with a variety of cannabinoid exposure procedures in primates.

2.6 Effects of cannabinoids on animal models of extinction and reinstatement of cocaine- seeking behavior

One of the major obstacles to successfully achieving withdrawal or abstinence from drug use stems from the difficulty of finding effective strategies against relapse to drug use that is thought to result from craving for the abused substance. Other than behavioral therapy, poten- tial pharmacological support might alleviate craving in abstinent individuals, thus reducing the likelihood of relapse. Although the validity of preclinical models of relapse to drug-seeking and drug-taking behaviors is still under discussion (147, 148, 149, 150), these procedures might give some useful information about which drugs and which brain areas might interfere with the processes that underlie relapse.

In the last years, as it has become increasingly clear that the endocannabinoid system plays a role in different aspects related to substance abuse, researchers have explored the involvement of the cannabinoid system in the neurobiology of relapse to drug-seeking (see citation 151 for review). In rats, when cocaine self-administration behavior was extinguished by substituting saline for cocaine injections (152), experimenter administration of increasing doses of delta-9- THC, 3 hours after withdrawal, failed to induce/reinstate cocaine-seeking behavior. However, administration of HU210, a synthetic cannabinoid agonist which has a higher affinity and greater intrinsic activity than delta-9-THC at CB1 receptors, provoked relapse to cocaine-seek- ing after a prolonged (14 days) withdrawal from cocaine (104). In addition to differences in treatment, the different outcome of these reports could be due to differences in abstinence strength, because in the first and second reports rats were abstinent from cocaine only for three hours (152) and 14 days (104), respectively. These different conditions might suggest time-re- lated adaptations of the brain systems involved in cocaine-seeking behaviors (153), with no influence of the endocannabinoid system during the early stages of withdrawal from cocaine. On the other hand, when the selective antagonists of cannabinoid CB1 receptors Rimonabant and AM251 were administered alone they did not reinstate cocaine-seeking behavior (104, 154), but both drugs reduced or inhibited cocaine-seeking behavior evoked by priming injec- tions of cocaine (104, 154). It is also interesting to note that Rimonabant reduced cocaine seek- ing behavior induced by cocaine associated cues, but not stress-induced reinstatement (104), suggesting the involvement of the endocannabinoid system only in some specific processes or pathways related to cocaine-seeking behavior. To this end, it is also interesting to note that AM251 did not block the DA-stimulating effects of cocaine in the NAc, but it attenuated the effects of cocaine on glutamate levels in the NAc, which might be a better neurochemical cor- relate of cocaine-seeking behavior (154).

3. Human studies

Though not overlapping with the purpose of this review, the “gateway drug” hypothesis of marijuana use is an interesting example of a link or association between cocaine dependence and cannabinoid agonists. Following this hypothesis, marijuana consumption could serve as a gateway drug for subsequent use and abuse of other illicit substances in a progression that can lead towards dependence on heroin, cocaine or other psychostimulants (155, 156, 157, 158). There is still an ample debate about the factors studied and taken into consideration, and the data obtained which support this theory (see for example citations 159, and 160). It is interest- ing to note, however, that independently from the factor/s that link marijuana use to subsequent abuse of other illegal substances, there are also studies that show a very high prevalence of marijuana use and dependence in cocaine dependent individuals (26, 161, 162, 163). Comor- bidity of cocaine dependence with other drug dependencies is also very common (25). Thus the occurrence of cannabis dependence in cocaine dependent subjects might suggest that indi- vidual vulnerability for a certain drug can be extended to other drugs (160), but also that com- mon risk factors (genetic, environmental, etc.) might jointly influence the individual drug lia- bility and the links between cannabis and other drug dependencies (163).

With regard to genetic risk factors, it is interesting to note that a human genetic variant of the cannabinoid receptor gene CNR1 has been associated with susceptibility of drug dependence in a non-Hispanic, Caucasian population (164). The association was stronger with intravenous drug use, and was greatest for cocaine, amphetamine, and marijuana dependence. Non-signifi- cant association data have been instead obtained in replication of the original findings by Comings and colleagues (165, 166). In a recent paper (31) these contrasting data are discussed as the result of the poor definition of the cannabinoid receptor CNR1 gene’s structure, regulation and variation. In the same report, indeed, the authors have shown an improved definition of the human cannabinoid receptor CNR1 gene’s locus and variants (31), and it has been also shown that haplotypes toward the 5′ end of the cannabinoid receptor CNR1 gene’s exons and introns differentiate between non-drug users and drug-abusers of European-American, African-Ameri- can, and Japanese subjects. Also, a recent report by Ballon and colleagues, 2006 (167), sup- ports the association of the cannabinoid receptor CNR1 gene polymorphism with predisposi- tion to cocaine dependence in an African-Caribbean population.

Another interesting association, from a therapeutic perspective, has been evaluated studying the relationship between marijuana use during cocaine abstinence and relapse to cocaine use. In a study by Labigalini et al., 1999 (168), the authors describe a clinical observation based on spontaneous accounts by Brazilian cocaine-crack abusers, undergoing their first psychiatric assessment, reporting use of cannabis in order to get relief from cocaine-withdrawal symp- toms. From a sample of 25 subjects, 68 percent ceased to use crack-cocaine during the study, which lasted 9 months. The small sample and the short period of time suggest a cautious ap- proach to the results outcome; further studies are warranted. A more recent study (169) in New York used a much larger sample of cocaine addicts (144 subjects), and examined whether cannabis use after discharge from inpatient treatment could affect relapse to cocaine use. Data from this study showed that about a third of the subjects used cannabis after discharge and that cannabis use substantially increased the risk of relapse to cocaine use, significantly reduced the achievement of sustained remission, and significantly increased relapse to cocaine use after sustained remission

(169). In agreement with this latter report is a preclinical investigation (de- scribed above, section 2.6) showing cannabinoid-induced reinstatement of cocaine seeking be- havior in rodents (104).

4. Summary and concluding remarks

Most of the scientific articles reviewed in the present manuscript have described studies of cannabinoid CB1 receptor agonists and antagonists tested against cocaine effects in preclinical models thought to be predictive of cocaine abuse. These studies have provided interesting re- sults, especially for the ability of cannabinoid antagonists, and Rimonabant in particular, to significantly counteract some of the reinforcing actions of cocaine (104, 143). Collectively, the studies suggest that a cannabinoid tone, impaired by cannabinoid antagonist administration, is indeed involved in many of the reinforcing effects of cocaine which are believed to be respon- sible for cocaine abuse and addiction. On the other hand, there are no studies available show- ing interactions of drugs acting as cannabinoid levels modulators/enhancers on cocaine-induced behaviors. These drugs affecting directly the endogenous cannabinoid tone could inter- fere with cocaine effects in these preclinical procedures and could substantially increase our knowledge about the cannabinoid-neurobiology related to cocaine dependence.

Suggestions about possible genetic predisposition/vulnerability to cocaine dependence from human studies due to variants of the cannabinoid receptor CNR1 gene have given more strength to the link between endocannabinoids and cocaine. Due to the widespread distribution of cannabinoid receptors in the brain, and their abundance in brain areas playing pivotal roles in drug abuse and addiction, the different expression and regulation of cannabinoid receptors induced by genetic differences (31) might be an important factor in the predisposition or vul- nerability to drug dependence. For this reason, the potential to directly interact with endo- cannabinoid tone in selected brain areas, an effect that can be obtained with endocannabinoid uptake inhibitors or metabolism blockers (4, 60, 62)(as shown also in genetically modified mice, 170), as compared to widespread actions of cannabinoid receptors agonists/antagonists, should be one of the next challenges in the research for medications able to counteract the abuse- and dependence-related behavioral/neurobiological effects of cocaine.

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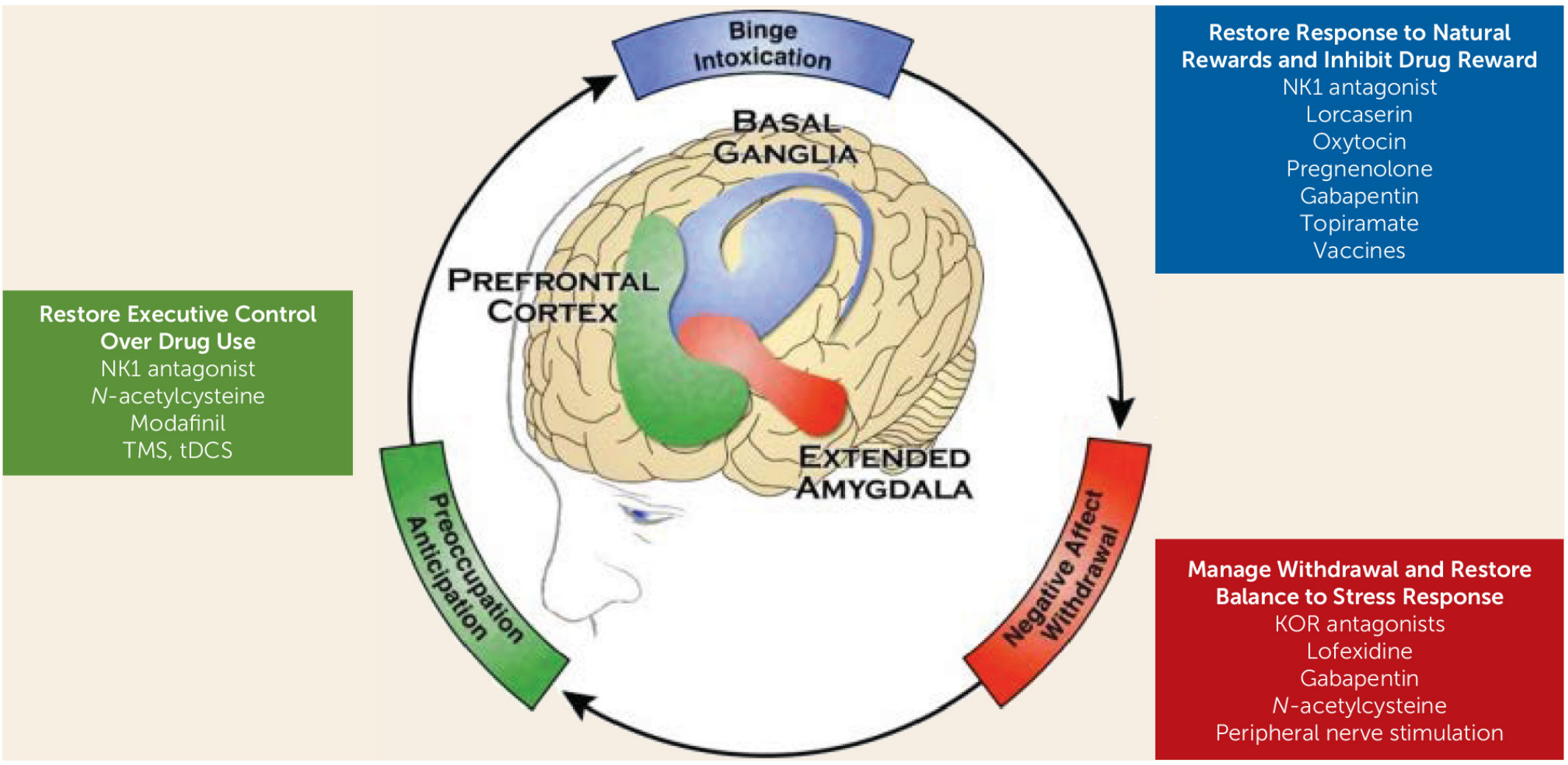

Modulation of the endocannabinoid system: vulnerability factor and new treatment target for stimulant addiction

Stéphanie Olière1, Antoine Jolette-Riopel 1, Stéphane Potvin2,3 and Didier Jutras-Aswad 1,2*

1 Addiction Psychiatry Research Unit, Research Center, Centre Hospitalier de l'Université de Montréal (CRCHUM), Montreal, QC, Canada

2 Department of Psychiatry, University of Montreal, Montreal, QC, Canada

3 Research Center, Institut Universitaire en Santé Mentale de Montréal, Montreal, QC, Canada

Edited by:

Elizabeth Clare Temple, University of

Ballarat, Australia

Reviewed by:
Elizabeth Clare Temple, University of Ballarat, Australia
Luigi Janiri, Università Cattolica del S. Cuore, Italy
*Correspondence:
Didier Jutras-Aswad, CRCHUM, St-Luc Hospital, Édouard-Asselin Pavilion, 264 René-Lévesque Blvd. East, Montreal, QC H2X 1P1, Canada
e-mail:
didier.jutras-aswad@umontreal.ca
Cannabis is one of the most widely used illicit substance among users of stimulants such as cocaine and amphetamines. Interestingly, increasing recent evidence points toward the involvement of the endocannabinoid system (ECBS) in the neurobiological processes related to stimulant addiction.This article presents an up-to-date review with deep insights into the pivotal role of the ECBS in the neurobiology of stimulant addiction and the effects of its modulation on addictive behaviors.This article aims to: (1) review the role of cannabis use and ECBS modulation in the neurobiological substrates of psychostimulant addiction and (2) evaluate the potential of cannabinoid-based pharmacological strategies to treat stimulant addiction. A growing number of studies support a critical role of the ECBS and its modulation by synthetic or natural cannabinoids in various neurobiological and behavioral aspects of stimulants addiction. Thus, cannabinoids modulate brain reward systems closely involved in stimulants addiction, and provide further evidence that the cannabinoid system could be explored as a potential drug discovery target for treating addiction across different classes of stimulants.
Keywords: addiction, stimulants, psychostimulants, cocaine, cannabis, cannabinoids or endocannabinoids

INTRODUCTION

Addiction to psychostimulants such as cocaine, amphetamine, and its derivatives [i.e., methamphetamine, N -methyl-3,4-methylenedioxymethamphetamine (MDMA)] is a significant global public health problem which affects many aspects of social and economic life. Worldwide, between 16 and 51 million people are users of these types of substances (1). Amphetamines have been identified as the second world's most widely used illicit drug after cannabis, with an annual prevalence ranging from 0.3 to 1.2% in the adult population. Methamphetamine consumption has increased dramatically the last years, especially in the western and mid-western parts of the United States, although there also appears to be an eastward trend in use (2). Over 15 million people worldwide are cocaine users and 5.9 million of them live in North America (3, 4). Although the prevalence of cocaine consumption has declined in the past decade, cocaine use increased in 2011; dependence to this drug remains a significant issue in North America, Western and Central Europe, particularly in metropolitan areas where crime and violence have increased (4). Given its association with high rates of mental and physical problems as well as premature mortality, cocaine abuse is still an unresolved medical and socio-economic concern which carries a heavy burden for abusers and their families alike (3, 5–7).

In recent decades, development of new treatments for psychostimulant addiction has been a major focus of multidisciplinary research efforts and have included molecular approaches, preclinical behavioral studies, and clinical trials. However, in spite of these research endeavors, no specific pharmacological therapy has been found to be truly effective in alleviating psychostimulant cessation symptoms like craving and anxiety, or to prevent relapse (8–11). Neuropharmacological agents such as antidepressants, anticonvulsants, and antipsychotics have been tested as treatments for cocaine dependence but these medications have yielded

negative clinical outcomes (12–14). Subsequent attempts at targeting other neurotransmitters such as the dopaminergic and γ-aminobutyric acid (GABA) systems (10, 15, 16), and developing a cocaine vaccine (17) to promote abstinence in cocaine-dependent individuals, have shown promising results but still require further investigation. Importantly, many clinical studies have focused on cocaine addiction rather than other psychostimulants such as amphetamines and methylphenidate. Whether the outcomes related to cocaine addiction can be applied to other psychostimulants remains unclear (18).

Given the need to better understand neurobiological mechanisms that underly psychostimulants addiction and to develop innovative treatment strategies, researchers have explored the involvement of specific neurotransmitter systems and brain structures in the motivational and addictive properties of this class of drugs. Increasing evidence indicates that the endocannabinoid system (ECBS) - a group of neuromodulatory lipids and receptors – plays a central role in various cognitive and physiological processes associated with addiction such as reward, stress responsiveness, and drug-related synaptic plasticity (19–21). The potential of ECBS modulation in treating stimulant addiction has recently been highlighted in human and animal studies investigating its effects on acquisition, maintenance, and relapse of drug-taking behavior. Moreover, endogenous and exogenous cannabinoids such as plant-derived cannabinoid ligands (i.e., Δ9-THC,

cannabidiol, CBD) modulate specific neurotransmitter systems which are also pharmacological targets for cocaine. Interestingly, cannabis is widely used by psychostimulant-dependent individuals (22); while it is recognized that components of the ECBS are implicated in psychostimulants, more specifically in cocaine-seeking behaviors, very few studies have focused on an understanding of the neural and behavioral effects of cannabis on psychostimulants use in humans.

The current review will focus on the neurobiological basis of the addictive process from psychostimulant initiation to drug abuse and addiction. The involvement of critical neurotransmitters and neural circuits underlying the pathological modifications at each of these stages will be highlighted with a specific attention given to the ECBS. In turn, this overview will serve as foundations to look at the ECBS as a specific target of pharmacotherapeutic interventions to reduce the addictive effects of psychostimulants.

NEUROBIOLOGY OF PSYCHOSTIMULANTS

Occasional use of psychostimulants like cocaine, at low doses, provokes a so-called “rush” (i.e., euphoria) in humans, giving them a sensation of vigilance and increased energy. Higher doses of cocaine induces symptoms described as “cocaine high,” which include enhancement of a euphoric sensation, an increase in motor activity, amplification of sensory perception, talkativeness, and suppression of appetite and thirst (23). Unfortunately, these positive subjective effects (i.e., euphorigenic state) are often followed by repetitive and frequent cocaine abuse which develops into addiction. Drug addiction is a chronically relapsing disorder characterized by loss of control over drug-seeking and the compulsive desire (referred as craving) to use drugs in spite of negative consequences (24). Drug cravings increase with exposure to drug and drug-related-cues, and in the context of emotional stress or negative moods (25, 26). Because addiction is a highly complex disorder, numerous studies have attempted to determine the mol-

ecular and cellular factors implicated in the pleasurable effects induced by drug consumption, and their role in the development of addictive behaviors (27–31).

NEUROTRANSMITTERS INVOLVED IN PROCESSES LEADING TO PSYCHOSTIMULANTS ADDICTION

Psychostimulants affect the central nervous system by modulating the mesocorticolimbic dopamine (DA) system which is involved in several physiological processes such as cognition, memory, and reward-driven learning (32). The mesocorticolimbic system, which has been found to play a role in drug reward and addiction, includes DA projections from cell bodies in the ventral tegmental area (VTA) to limbic structures such as the nucleus accumbens (NAc) (33), amygdala in the forebrain (34, 35), hippocampus (36), and to cortical areas such as the prefontal cortex (PFC), including the orbitofrontal cortex (OFC) and anterior cingulate (AC) (37). Psychostimulants exert their effects on the CNS via a number of mechanisms; cocaine, amphetamines, methamphetamines, and methylphenidate alter normal DA receptor functions by binding the dopamine transporter (DAT) and forming a complex that blocks the transporter's function. The psychostimulant/DAT complex inhibits DA reuptake into the presynaptic nerve terminal, leading to an excess of DA in the synaptic cleft within the NAc – recognized as the center of the rewarding process (38–40). This phenomenon results in an increased and prolonged post-synaptic effect of dopaminergic signaling at DA receptors on the receiving neuron (30, 31). However, unlike cocaine, which interferes mainly with plasma membrane transporters, other psychostimulants modulate the CNS through a host of mechanisms. First, methamphetamines and amphetamines act as substrate-type releasers (41, 42) to enhance DA efflux. These substrate-type releasers have two modes of action: (i) they reverse the process of transporter-mediated exchange by interacting with specific transporter proteins which are subsequently brought into the cytoplasm of the nerve terminal; (ii) they also increase cytoplasmic levels of DA by interfering with vesicular storage (43, 44). Moreover, these drugs increase cytosolic DA levels by shutting-down the activity of the monoamine oxidase (MAO) – an important enzyme for the catabolism of monoaminergic neurotransmitters. Finally, psychostimulants also enhance the activity and expression of the tyrosine hydroxylase (TH), the DA-synthesizing enzyme [reviewed in Ref. (45)]. However, exactly how these high levels of DA in the NAc mediates drug reward remains partially understood.

Even though DA has been identified as one of the primary mechanisms involved in drug reinforcement initiation, studies reveal that mice lacking the gene expressing the DAT continue to self-administer cocaine (46, 47). Interestingly, several reports have suggested the indirect implication of other neurotransmitter systems [i.e., serotonin (5-HT), norepinephrine (NE), glutamate (GLU),GABA,opioid peptides,and endocannabinoids (48–50)] in the incentive sensitization and reinforcing effects of psychostimulants (29, 51). Indeed, psychostimulants also reduce 5-HT and norepinephrine NE reuptake, which in turn leads to an increase in extracellular monoamines concentrations and contributes to the rewarding subjective feelings mediated by these drugs (42, 52–54). Surprisingly, knock-down of NET, or SERT, or NET/SERT genes does not abolish but rather potentiates the rewarding or aversive effects of cocaine (55, 56). Recently, further lines of evidence have suggested that NE plays a role in the reinstatement of drug seeking, although it does not influence the maintenance phase of cocaine self-administration (SA) (57–59). Blockage of NE cognate recep-

tors – α1-adrenergic receptors (α1ARs) and β-adrenergic receptors (βARs) – in a mice model of addiction diminishes cocaine-primed and foot shock-induced reinstatement respectively, whereas inhibition of both receptors reduces cue-induced reinstatement (60, 61).

Long-term use of psychostimulants leads to homeostatic dysregulation of normal (i.e., without cocaine) dopaminergic signaling. This hypo-dopaminergic state contributes to the appearance of some withdrawal symptoms (i.e., depressive mood disorders), often observed in abstinent psychostimulant addicts, and, also the maintenance of drug-use behaviors. Similarly, withdrawal symptoms from chronic cocaine use have been also associated with cocaine-induced alterations in 5-HT neurotransmission. Interestingly, rodent studies show that enhancement of serotonergic transmission in the NAc through administration of exogenous 5-HT served to offset the DA deficit caused by cocaine withdrawal (62); indeed, accumulating studies suggest that increasing brain 5-HT activity could reduce the behavioral-stimulant and reinforcing properties of psychostimulants (reviewed in Ref. (44)]. Thus,

modulation of 5-HT and DA levels might sensitize an important brain reward circuit to the reinforcing effects of psychostimulants contributing to the intractable nature of addiction and relapse.

The glutamatergic system is another important neuronal substrate of behaviors induced by drugs of abuse (63,64). Indeed,GLU is an excitatory neurotransmitter essential to numerous processes including neuroplasticity, linked to long-term potentiation (LTP), long-term depression (LTD), extinction, and reward-related learning (65–68). Like DA, GLU levels in the NAc core decrease during the early phase of cocaine abstinence (69, 70), whereas both stress and drug-induced reinstatement of cocaine-seeking are associated with an increase of extracellular GLU levels in the NAc in rodents (63, 64, 70–73). Thus, the data suggests that both a decrease in basal GLU transmission and an enhanced GLU response may constitute a neurobiological substrate of cocaine-, cocaine-associated cue-triggered relapse.

While discovery of numerous neurotransmitter systems have yielded significant advances in defining psychostimulants' effects on the brain neurochemistry, the precise mechanisms underlying their role in addictive behaviors are not as straightforward. While DA and GLU appear to be critical in the development and persistence of stimulant addictive behaviors, a growing body of evidence points toward the impact of other neurotransmission systems, including the ECBS, in various physiological and behavioral processes associated with psychostimulant addiction, through both DA/GLU related and unrelated mechanisms. An overview of this evidence will be presented in Section "The Endocannabinoid System," with a particular focus on the potential exogenous and endogenous cannabinoids influence on psychostimulant reinforcement, drug-related synaptic plasticity, and drug-seeking behavior.

NEURAL REGIONS INVOLVED IN ADDICTION PROCESS TO PSYCHOSTIMULANTS

A central challenge in addiction research is understanding the neurobiological substrates involved in drug-taking behavior. Over the last two decades, neuroimaging has provided substantial insight into that question by: (i) allowing researchers to investigate the roles of different neural regions in drug-induced euphoria and subsequent craving; (ii) enabling the gathering of tremen-

dous information regarding the neurochemical and physiological adaptations of the brain during the addiction process.

Brain imaging studies of subjects addicted to psychostimulants indicate that the NAc – known to play a fundamental role in goal-directed behaviors (74) – is organized into two functionally distinct sub-compartments termed the shell and core (33). The shell and the VTA are critical in inducing motivational salience and responding to novel rewarding stimuli (75). The core mediates the expression of learned behaviors, and receives glutamatergic afferents from the PFC (33, 75). DA release into the core occurs in response to cues predicting a motivating event (76, 77). The NAc receives information regarding motivationally relevant events from the VTA, amygdala, hippocampus, and PFC, and responds by providing output to brain circuits which modulate the expression of the behavioral response (e.g., to seek the drug or not) (78). Chronic exposure to psychostimulants leads to the dysregulation of the mesolimbic circuitry, which in turn enhances the motivation to take drugs and decreases the ability to regulate the behavioral response to drug cues (33, 74).

The numerous neuroimaging methods used to study the chronic effects of psychostimulants on the brains of drug-addicted individuals have consistently found abnormalities in both cortical and subcortical neural areas (37, 79). More specifically, chronic exposure to psychostimulants causes functional alterations within frontal brain areas, including the dorsolateral prefrontal cortex (DLPFC), the OFC, and the anterior cingulate cortex (ACC) involved in goal identification; selection (80); decision making; impulsivity; behavioral inhibition (81), and assessment of consequences (82), respectively. It has been proposed that abnormalities within these three PFC-striatothalamic circuits play a central role in emotional response to drug cues, craving, compulsive drug-seeking, and relapse (26, 35, 83–85). Moreover, structural magnetic resonance imaging (structural MRI) studies associate chronic use of psychostimulants with alterations in white-matter integrity and gray-matter volume, which are strongly correlated with lower abstinence-based outcomes (86) and drug-induced compulsivity, decision making, and attention impairments in cocaine-dependent subjects, respectively (85). Furthermore, exposure to emotional distress and aversive stimuli also activates the cortico-limbic circuits, including prefrontal, AC, middle frontal, and orbitofrontal regions, limbic and paralimbic structures such as the amygdala, hippocampus, parahippocampal gyrus, fusiform gyrus, and other midbrain areas, but not the ventral striatum (87, 88). Overall, the data shows that chronic use of psychostimulants modulates a set of neural regions implicated in stress, emotions, impulsivity, and reward processing control which precipitate relapse in drug-abstinent individuals.

THE ENDOCANNABINOID SYSTEM

Though the significant role played by various neurotransmitters, genetic factors and specific brain structures in reinforcing the properties of psychostimulants has been established, the common mechanisms underlying the development of addictive behaviors have yet to be fully elucidated. A growing body of evidence points to the involvement of the ECBS in the acquisition and maintenance of drug-taking behaviors and in various physiological, as well as behavioral processes associated with addiction. Interestingly, a characteristic of psychostimulants abuse is the concurrent consumption of other substances including delta-9-tetrahydrocannabinol (Δ9-THC) – the main cannabinoid found

in cannabis [reviewed in Ref. (89)]. This poly-substance pattern of use has prompted researchers to investigate the potential interaction with, and effect of, these drug on neuropsycho-biological processes related to addiction. For example, some studies reveal that cannabis consumption enhances the incentive to use cocaine in individuals dependent on both, while others suggested that cannabis reduced withdrawal symptoms in abstinent cocaine-addicted subjects (22, 90, 91). Although it is recognized that components of the ECBS are involved in cocaine-seeking behaviors, very few studies have focused on understanding the neural and behavioral effects of endogenous and exogenous cannabinoids on psychostimulant use. In this section, we will first provide an overview of the ECBS, and then focus on recent findings

pointing toward a role of the ECBS in the circuitry underlying psychostimulant addiction.

OVERVIEW

The ECBS consists of a family of lipid signaling molecules referred to as endocannabinoids, their cognate receptors and specific metabolic enzymes which are responsible for degradation of the endocannabinoids – anandamide (AEA) and 2-arachidonoylglycerol (2-AG). The neurobiological properties of endocannabinoids are complex, but it is now well established that they modulate a wide diversity of physiological processes including pain and inflammation, immune responses, food intake, synaptic transmission, cognition, reward, and motor activity (92). Endocannabinoids also influence mechanisms involved in addiction and relapse.

RECEPTORS

There are currently two well described subtypes of cannabinoid receptors, termed CB1 and CB2, which differ in their signaling mechanisms and tissue distribution. Even though CB1 receptors are considered the most abundant and widely distributed G-protein-coupled receptors found in the CNS, they are also present in peripheral organs and tissues (i.e., endocrine glands, leukocytes, spleen, heart, and gastrointestinal tracts, etc.) (93–97). CB1 receptors are localized in TH-expressing neurons, probably dopaminergic neurons of the NAc, VTA, striatum, and pyriform cortex, suggesting that the ECBS may directly influence dopaminergic reward mechanisms. In addition, CB1 receptors are expressed in other neural regions related to reward, motivation and memory processing (i.e., basolateral amygdala, hippocampus, and cerebral cortex), movement (i.e., basal ganglia, cerebellum), pain modulation (i.e., certain parts of the spinal cord, periaqueductal gray). Endocannabinoids induce LTD of the inhibitory synapses in the hippocampus, contributing to the synaptic plasticity involved in the learning processes related to addictive behaviors. CB1 receptors are confined at the terminals of central and peripheral nerves, where they inhibit the release of excitatory and inhibitory neurotransmitters (release on command, retrograde signaling) (98–100). Thus, the activation of CB1 receptors protects the nervous system from over-activation or over-inhibition by neurotransmitters and thereby promotes the latter's prominent role in anxiety, depression, cognition, addiction, motor function, feeding behavior, and pain (101). CB2 receptors are mainly found in immune cells (i.e., spleen, tonsils, and thymus gland) (102–104), although recent experimental data indicate CB2 receptors expression in the cerebellum, brainstem, and cortex (105–107) as well as activated microglial within the CNS (108–110). Simulation of CB2 receptors on microglia modulates the neuro-inflammatory response by

regulating cytokines release in the brain (111–113).

Increasing evidence points toward the existence of additional cannabinoid receptors subtypes in the CNS. Indeed, recent preclinical studies suggest the persistence of cannabinoid-like properties after cannabinoid agonists have been administered to mice lacking CB1 and CB2 receptors (CB1–/–and CB2–/–) in neuronal subpopulations. This indicates that these agonists recognize non-CB1/CB2 cannabinoid receptors (114–117). Among these receptors, the orphan G-protein-coupled receptors modulate the ECBS. GPR55 specifically is found in the striatum and to a lesser extent in the hippocampus, the thalamus and the cerebellum (118). GPR55 is phylogenetically different from CB1 and CB2 receptors, in that it is activated by the CB1 antagonists – rimonabant and AM251 – but blocked by the cannabinoid agonist CP55, 940 (119–121). Thus, GPR55 is considered as a non-CB receptor with a binding site for cannabinoid ligands [reviewed in Ref. (122)]. Though a recent study from Rusakov's group suggests that GPR55 enhances neurotransmitters release at central synapses (123), further studies are required to confirm its neurophysiological function.

The actions of endocannabinoids are not only restricted to the CB1, CB2, and GPR receptors. Transient receptor potential (TRP) receptors have also been identified as sites of endocannabinoid interaction. Exogenous and endogenous cannabinoids interact with at least five TRP receptors (124); AEA binds to the transient receptor vanilloid potential 1 (TRVP1) with low affinity. TRVP1 is found on sensory neurons, where they are partly coexpressed with CB1 receptor, but also in several central nuclei including the hypothalamus and basal ganglia, the hippocampus and cerebellum (125). The efficacy and potency of AEA at TRVP1 is increased when the AEA degrading enzyme FAAH (fatty acid amide hydrolase) is suppressed (126–128). Surprisingly, pharmacological or genetic inhibition of FAAH enhances AEA, but decreases 2-AG levels via TRVP1 receptors (129). Interestingly, both endocannabinoids AEA and 2-AG decrease the excitatory GLU and the inhibitory GABAergic inputs to striatal neurons (130, 131). Therefore, it is likely that the potential of AEA to reduce 2-AG levels by activating TRVP1 receptors might represent a mechanism to integrate excitatory and inhibitory inputs in the basal ganglia.

ENDOCANNABINOIDS AND THEIR METABOLIZING ENZYMES

In the CNS, endocannabinoids mediate forms of short-term synaptic plasticity known as depolarization-induced suppression of inhibition (DSI) (132, 133) and depolarization-induced suppression of excitation (DSE) (134). Thus, endocannabinoids are considered as retrograde messengers that neuromodulate diverse physiological processes. AEA and 2-AG are the two most characterized endocannabinoids (135, 136), although other studies have identified of additional endocannabinoids such as 2-arachidonylglyceryl ether (noladin ether) (137), N -arachidonoyl-dopamine (NADA) (128), and O-arachidonoyl-ethanolamine (virodhamine) (138). However, the physiological functions of these endocannabinoids are still being investigated. While 2-AG acts as a full agonist at CB1 and CB2 receptors, AEA behaves as a partial agonist at both receptors subtypes and can also interact with GPR55 and TRVP1 receptors.

Unlike other neurotransmitters, AEA and 2-AG are not synthesized and stored in the nerve cells. Rather, they are produced on an "as needed" basis by their membrane lipid precursors in a Ca2+ dependent fashion (133, 139). Although additional studies are needed to ascertain the exact role of the N-acylphosphatidylethanolamine phospho-lipase D in the ECBS, it

has been proposed that this enzyme might play a significant role in the synthesis of AEA (140). The enzyme responsible for 2-AG synthesis is the diacylglycerol lipase alpha (141). Upon depolarization of post-synaptic neurons, the endocannabinoids released into the synaptic cleft bind to and activate the presynaptic CB1 receptors, which in turn suppress the release of both excitatory and inhibitory

different neurotransmitters [see for review (142)]. Then, AEA and 2-AG are rapidly deactivated by cellular reuptake into both neurons and glial cells and metabolized by specific enzymes (143). AEA can be metabolized by either the FAAH (144), or monoacylglycerol lipase (MAGL), which degrades specifically 2-AG (145). In addition to MAGL, recent studies have suggested that the enzymes ABHD6 and ABHD12 could also be involved in 2-AG metabolism (146, 147). FAAH is over-expressed in the CNS and FAAH-positive neurons are localized in proximity to CB1 receptor-containing terminals, underlining the role for this enzyme in endocannabinoids inhibition (148). Thus, selective inhibition of FAAH (149) and MAGL (150) can prolong the effects of endocannabinoids.

Pre-clinical studies have demonstrated that pharmacological inhibition of FAHH with URB597 (149) or PF-3845 compounds (151) induced-anxiolytic-like effects (152, 153) and anti-nociceptive properties in mice (152, 154). Inhibition of MAGL with JZL184 inhibitor causes analgesia, hypothermia, and hypomotility (155). However, chronic exposure to JZL184 impairs endocannabinoid-mediated synaptic plasticity in mouse hippocampus and cerebellum via 2-AG upregulation. It also induces tolerance to the analgesic effects, physical dependence, and persistent activation as well as desensitization of brain CB1 receptors (156). Surprisingly, MAGL knockout mice show enhanced learning behavior and have normal locomotor activity, suggesting the possible role of MAGL in cognitive function (157, 158).

EXOGENOUS CANNABINOIDS: Δ9-THC VS. CBD

Cannabis is the world's most commonly used illicit drug (159, 160). Between 119 and 224 million people are cannabis users worldwide (4). Cannabis contains over 85 different chemical substances unique to the plant and termed phytocannabinoids. Among them, Δ9-tetrahydrocannabinol (Δ9-THC) and CBD are the two main components of cannabis, which has been used for thousands of years for both recreational and medicinal purposes.

Most studies regarding cannabis properties have focused on Δ9-THC, which is the main psychoactive constituent in cannabis extracts (161). Although Δ9-THC possesses a number of therapeutic effects (e.g., on pain, spasms, inflammation), its negative impact on the CNS has been highlighted in several clinical studies on subjects smoking cannabis, documenting impulsive behavior, cognitive impairment, consumption of addictive substances, and psychiatric disorders (e.g., schizophrenia, depression, and anxiety) (162–165). For example, Δ9-THC has been shown to induce psychotic-like and anxiogenic effects when administered intravenously to healthy subjects (166,167). Other experimental studies revealed that Δ9-THC injection in animal models causes hypolocomotion, catalepsy, antinociception, and hypothermia (168). Pharmacological studies in animal models suggest that not all therapeutic effects related to cannabis administration can be ascribed to Δ9-THC [reviewed in Ref. (169)]. Indeed, CBD – the second most abundant cannabinoid found in cannabis – acts as an antidepressant and possesses anticonvulsant, antiemetic, anxi-

olytic, and sleep-promoting as well as neuroprotective properties in humans (160, 170–176). CBD mediates its neuropharmacological properties by acting as an inverse agonist on CB1 and CB2 receptors (177, 178); it also stimulates the TRVP1 and TRVP2 (179) which serve as so-called ionotropic cannabinoid receptors. In addition, CBD inhibits FAAH, the main catabolic enzyme that alters the hydrolysis of the endogenous cannabinoid neurotransmitter AEA (180) (see above section), and is also an antagonist at the putative GPR55 receptor. The clinical association of the modulation of the ECBS by CBD remains to be fully investigated; this effect could arguably be related to DA uptake inhibition (181). Interestingly, ECBS interacts closely with other neurobiological structures which are implicated in the neural adaptations observed during chronic use of drugs and vulnerability to addiction. For example, CBD plays a role in the modulation of extracellular levels of DA (182) as well as μ and δ opioid receptors (183); it increases adenosine signaling through inhibition of uptake (184). Moreover, μ opioid and CB1 receptors colocalized within neural regions are known to modulate reward, goal-directed behavior, and habit formation relevant to addiction including striatal output projection neurons of the NAc and dorsal striatum (185, 186). While further studies are required to better understand the impact of CBD on GLU neurotransmission, its protective effects on GLU toxicity (187) and its psychopharmacologic interaction with ketamine (188), a N -methyl-d-aspartic (NMDA) receptor antagonist, are well documented. CBD activates also the serotoninergic receptors 5-HT1A (5-hydroxytryptamine) (171, 176, 189–193), which in turn diminishes vulnerability to stress and has anxiolytic-like effects in animal models (170, 172, 189, 190, 192–195). Similar results were observed in humans, where CBD administration decreases autonomic arousal and subjective anxiety (196). Interestingly, these anxiolytic effects have been linked to the modulation of core regions involved in the “emotional brain,” including limbic system structures such as the AMG and the ACC (197, 198). CBD’s anxiolytic effects were further confirmed by a study indicating that the effective connectivity between ACC and AMG is attenuated during the emotional processing of fearful faces, while resting activity of the left parahippocampus gyrus is increased. (196, 199). Remarkably, these neural structures are activated during drug craving in cocaine addiction (197, 200). It also decreases compulsive behaviors in rodents, which is hypothesized to be related to CB1-related mechanisms (201, 202).

While CBD has neuroprotective properties (187, 203, 204) and Δ9-THC administration have been shown to cause neurotoxic effects (205), these opposing properties have been highlighted in brain imaging studies where Δ9-THC and CBD activate different brain regions during tasks engaging verbal memory (206, 207), response inhibition (208), and emotional processing (196, 209–211). When given at appropriate doses, CBD counteracts Δ9
-
THC properties. Thus, CBD can modulate the functional effects of Δ9-THC (177, 178). Pre-clinical studies demonstrate that CBD decreases Δ9-THC-induced conditioned place aversion and social interaction of on operant behavior model (212, 213). In addition, CBD diminishes Δ9-THC-induced anxiety and psychotic-like symptoms in humans (214, 215). Together, this data clearly suggests that CBD limits Δ9-THC adverse effects. Thus, administered together, CBD might increase Δ9-THC clinical efficacy (216, 217). It has been established that unlike, Δ9-THC, CBD possesses therapeutic properties that could reduce withdrawal symptoms

often present in individuals with addictive disorders (e.g., anxiety, psychotic, mood symptoms, insomnia, and pain). For example, a recent pre-clinical study from Hurd's group aimed at assessing

the effects of cannabinoids on opioid-seeking behaviors in rats indicates that while Δ9-THC potentiates heroin SA, CBD inhibits cue-induced heroin-seeking behaviors for up to 2 weeks following the last administration (218). In addition, CBD is well tolerated and has no gross effects on motor function (such as locomotor activity). CBD is also protects against damages caused by various substances; it reverses binge ethanol-induced neurotoxicity (219) and mitigates the cardiac effects of Δ9-THC (220, 221). Together this data illustrates the different, and sometimes opposite, neurobiological properties of the two main constituents of cannabis – CBD and Δ9-THC – that are linked to neural circuits which might play significant roles in addiction disorders. However, while numerous studies have highlighted the participation of the ECBS in the rewarding and addictive properties of drugs of abuse such as opioids, nicotine, and alcohol over the last decades, relatively few studies have focus on the impact of this system on addiction to psychostimulants.

INTERACTION OF THE eCBS WITH BIOLOGICAL AND BEHAVIORAL CORRELATES OF PSYCHOSTIMULANTS ADDICTION

HUMAN STUDIES

Human studies aimed at understanding the interaction of the ECBS with biological and behavioral correlates of addiction to psychostimulants have mostly focused on ECBS-related risk factors leading to drug dependence. Interestingly, cannabis use is strongly associated with the abuse and/or dependence of several class of drugs including psychostimulants such as cocaine (222). Moreover, exogenous cannabinoids have been shown to modulate the acute rewarding effects of cocaine. These lines of evidence may suggest an association between ECBS and liability to psychostimulant by pointing toward a possible involvement of the ECBS in the motivational effects mediated by psychostimulants (223) [reviewed in Ref. (224)]. Based on these observations, scientific efforts have been devoted to investigate the influence of various genetic (e.g., ECBS-related genes) and environmental characteristics (e.g., previous or current exposure to cannabinoid agonists) in individual progression from occasional use to psychostimulant addiction.

"The gateway theory" and addiction to psychostimulants

Association of prior or concomitant cannabis consumption with other illicit drugs including psychostimulants such as methamphetamine and cocaine, forms the basis of a well-known hypothesis –" the gateway theory,"which suggests a causal role for cannabis in the development of subsequent drug use and addiction (225). While data indicate that smoking cannabis is positively associated with cocaine consumption, it would be inappropriate to assume that cannabis per se leads to cocaine use. A study from Lynskey et al. in human twins reveals that early cannabis use in life increases the odds of subsequent cocaine use, supporting the causative model of the "gateway theory." However, results of this study have been refuted by Kandel et al. (226) which argues that several additional genetic, social, and environmental factors, such as life experiences, might link cannabis use with subsequent cocaine consumption (227, 228). Actual neurobiological causal mechanisms underlying this "gateway theory" remain mostly unidentified. Interestingly,

Tomasiewicz and colleagues show that Δ9-THC exposure induces epigenetic dysregulation of the endogenous opioid proenkephalin in adolescents; these findings indicate that cannabis exposure, in and of itself, can be considered as a risk factor that acts "above the genome" and can "write" on the existing epigenetic background of adolescent neurodevelopment. Thus, in adolescents, Δ9-THC exposure-mediated epigenetic effects may act in concert with other environmental or social factors to augment future behavioral responses to drugs of abuse via stable and long-term regulation of genes at the transcriptional level. However, while these data establish a direct link between Δ9-THC-induced changes in proenkephalin expression and susceptibility to opiate drugs, no studies have confirmed that this mechanism can be applied to psychostimulants (229).

Genetic determinants of the ECBS and psychostimulant addiction

It is worth mentioning that not every subject who experiences the pleasurable effects of psychostimulants will become a chronic user. Indeed it is more likely that additional factors such as: (1) genetic variabilities (e.g., polymorphisms in the catechol O-methyltransferase gene (Val158met) and in the serotonin transporter gene (5-HTTLPR) (230, 231); (2) monoamine receptors deficiency – either genetically or as a result of their drug excesses – also contribute to the psychostimulants addiction process (232–235) (see Table 1). In the ECBS, different genetic variants of the CB1 receptors – CNR1 – and FAAH genes have been associated with increased susceptibility to drug addiction. Indeed, genetic analyses demonstrate that the CNR1 gene exhibits elevated numbers of (AAT)n triplet repetition in a sample of 192 non-Hispanic Caucasian subjects. Interestingly, this CNR1 polymorphism increases the risk of intravenous drug use in this population, with strongest correlation observed in cocaine, amphetamine, and marijuana dependence (236). Similarly, a study from Ballon and colleagues shows that detection of this CNR1 polymorphism in a sample of 142 African-Caribbean individuals predisposed them to cocaine addiction (237). Unfortunately, while single sequence repetitions can alter transcriptional rates and thereby induced gene overexpression or silencing (238), the functional nature of the microsatellite polymorphism triplet repetition (AAT)n in modulating CNR1 gene expression remains blurred (239). It has been hypothesized that the presence of long alleles with high numbers of AAT triplets alter CNR1 transcriptional gene expression, ultimately leading to low levels of CNR1 protein synthesis (240). A recent meta-analysis of 11 studies aimed at investigating the contribution of three CNR1 polymorphisms (rs1049353, rs806379, and the AAT triplet repetitions) to drug dependence vulnerability confirmed the presence of (AAT)n repeats, but only in the Caucasian population [reviewed in Ref. (239)]. Unfortunately, the effect of the three CNR1 polymorphisms appeared to be insignificant and showed high heterogeneity. Important caveats have to be considered when looking at these studies. First, the ethnicity of the different subjects may prove important, as some studies included several ethnic groups in their samples, and in some cases, these groups were not even mentioned (241, 242). Some reports also examined CNR1 gene polymorphisms in connection with a different phenotype or stage of drug addiction such as craving, drug consumption, dependence, or drug withdrawal

Table 1 | ECBS and factors contributing to vulnerability to psychostimulants in humans.

Aspect Conclusion Reference

Genetic risks factors CNR1 (AAT)n repeat polymorphism associated with IV drug use, including amphetamine and cocaine, in a non-Hispanic Caucasian population and with cocaine dependence in an African-Caribbean population
Comings et al. (236), Ballon et al. (237)
CNR1 gene single nucleotide polymorphisms associated with cocaine addiction in an African-American population
Clarke et al. (244)
FAAH gene mis-sense mutation associated with drug dependence Sipe et al. (245), Flanagan et al. (246)
Cannabis effect in addiction to psychostimulants
Self-reported of cannabis smoking by crack-cocaine abusers alleviates withdrawal symptoms and drug-craving
Labigalini et al. (90)
Post-discharge use of cannabis by American cocaine addicts increases risk of relapse
Aharonovich et al. (91)
Cannabis use correlates with syringe sharing in injection drug users Jutras-Aswad et al. (22)
Recent cannabis use decreases activation of frontal cortices area during emotional stress stimulation in cocaine-dependent individuals
Li et al. (247)

(243). Furthermore, a detailed description of the repercussions of CNR1 polymorphisms on CB1 function from a neurobiological standpoint is lacking from the reviewed studies.

Polymorphisms in the gene coding for the endocannabinoid-inactivating enzyme FAAH may constitute another risk factor for problematic drug use, as described by initial reports identifying C385A, a mis-sense single nucleotide polymorphism (SNP) causing reduced FAAH enzymatic activity (245, 246). Indeed, a study from Sipe et al. reveals significant association between C385A SNP and street drug abuse in a sample of 1737 Caucasian subjects with addictive disorders. Neuroimaging studies combined with genetic analysis reveal that low FAAH activity enhances AEA protein expression levels which, in turn, modulate brain regions implicated in drug addiction and reward circuitry such as the OFC, AC gyrus, and NAc (242). Additional neuroimaging studies show that C385A carriers exhibit increased ventral striatal reactivity – a correlate for heightened impulsivity and reward sensitivity. C385A carriers display low threat-related amygdala reactivity – a pattern observed in individuals with high familial risk of alcoholism. Moreover, C385A polymorphism-reduced FAAH functional activity increases risk-taking behavior associated with addiction through abnormal impulsivity and threat perception [reviewed in Ref. (224, 243)]. Contribution of SNPs that modulate FAAH functions to stimulant addiction remain to be explored as the aforementioned data were not obtained in individuals specifically addicted to stimulants.

Effect of exogenous cannabinoids on psychostimulant reward

As mentioned previously (see The Endocannabinoid System), an intriguing characteristic of psychostimulants abuse is the concurrent consumption of cannabis. Parallel to studies on the long-term effects of cannabis exposure on subsequent psychostimulant use, researchers also examined the acute rewarding effects of cannabis use on concurrent psychostimulant addiction (91, 222). However, studies aimed at investigating such interactions are sparse. Conflicting results from Foltin et al. and Lukas et al. provide evidence that cannabinoids modulate cocaine-mediated euphoric actions. First, data from Foltin and colleagues show that human volunteers who smoked cannabis prior to intravenous cocaine experience a prolongation of the “high” sensation (248). Second, a study from Lukas and colleagues reveals that smoking Δ9-THC, 30 min prior

to intranasal cocaine decreases the latency to onset of cocaine-induced euphoria significantly, from 1.87 to 0.53 min, as well as the duration of cocaine-induced dysphoria, from 2.1 to 0.5 min (249). Interestingly, when both drugs are administered concomitantly, no changes are observed in cocaine- and Δ9-THC-induced positive subjective properties. Furthermore,Δ9-THC increases the peak plasma levels and bioavailability of cocaine considerably. This increase might be the result of Δ9-THC-induced vasodilation of the nasal mucosa which, in turn, reduces cocaine-induced vasoconstriction, thereby increasing cocaine's absorption. In addition, the discrepancies between these two studies might be also due to pharmacodynamic mechanisms including differences in cocaine absorption or in the ratio of CBD/Δ9-THC levels found in the type of cannabis used for each study.

Using fMRI technology combined with script-guided imagery paradigm in which subjects imagined being in a real-life stressful situation, Rajita Sinha's group found that cannabis abuse contributed to stress-induced blood-oxygen-level-dependent (BOLD) contrast in a group of cocaine-dependent individuals. More specifically, cannabis consumption decreases emotional stress cue-induced frontal and cingulate activation in cocaine-dependent individuals (247). These findings suggest an abnormal cognitive control mechanism during affective processing in association with heavy cannabis use. An important caveat to consider in the latter study is that cocaine-dependent individuals were abstinent for several weeks prior to the neuroimagery session and were not current users of cannabis. Thus the study did not allow to examine the acute effect of cannabis on neural and behavioral responses. However, the fact that this cannabis-induced alteration in stress-response can be translated to cocaine craving and relapse vulnerability has definitely piqued further interest, and initial data on this matter already exists. Indeed, the effects of cannabis consumption on abstinence and relapse to cocaine use have been provided in a

study from Labigalini and colleagues, in which 25 cocaine-crack dependent individuals reported to smoke cannabis in order to get relief from abstinence mediated-cocaine-withdrawal symptoms. From this sample, 68% of addicts achieved crack-cocaine cessation while using cannabis during the 9 months duration of the study (90). However, the self-reported nature of this study and its limited duration suggest cautiousness in interpreting its outcome. In a more recent study, Aharonovich et al. drew opposite conclusions on the consequences of smoking cannabis on cocaine relapse. In this study, researchers investigated whether cannabis use after the discharge of 144 drug-addicts from inpatient treatment program could help them to maintain abstinence and thereby preventing relapse to cocaine use. Results from this study suggest that smoking cannabis reduced the achievement of sustained remission and increased relapse to cocaine use (91) (see Table 1). Surprisingly, a study from Jutras-Aswad et al. supports the assumption that irregular cannabis use increases risky behaviors (syringe sharing) of cocaine and opioid users, as opposed to regular cannabis use, suggesting a complex dose-effect relationship between cannabis and addictive behaviors (22). The possibility that cannabis use by recently abstinent cocaine-dependent individuals influences relapse to drug and other related behaviors remains poorly documented.

ANIMAL STUDIES

Over the last decades, development of animal models have allowed

a better understanding of psychostimulant effects and addiction-related behaviors. These studies would not be available through clinical studies for ethical and practical reasons. Notably, invasive measures such as catheter installation for drug administration and surgical brain procedures for assessment of drug-induced neurobiological changes, as well as strictly controlled conditioning protocols involving restrictive environments, have extended the knowledge of psychostimulants effect on neurotransmission. These methods have also allowed observations of specific behavioral aspects of psychostimulant addiction. Thus, studies on animal models of psychostimulants abuse have provided tremendous insights on the role of ECBS in various aspects of psychostimulant addiction, spanning from drug reward, acquisition, and relapse.

Influence of ECBS on psychostimulants-induced behavioral and reinforcing effects

As mentioned above (see Neurobiology of Psychostimulants), substantial evidence indicates that behavioral and addictive properties of psychostimulants come from the interactions of psychostimulants with brain monoamines. Specifically, increase of extracellular levels of DA through promotion of DA release by amphetamine and MDMA, as well as inhibition of DA reuptake by cocaine, represent the primary mechanisms involved in rewarding effects mediated by psychostimulants (224). In animal models of intracranial self-stimulation (ICSS), the rewarding properties of drugs of abuse typically translate into lowering of the so-called reward threshold established after operant training [see Ref. (250) for description]. Initial experiments showed no effect of CB1 antagonist SR141716A on cocaine's ability to lower ICSS threshold in rats (251), although careful data analysis suggests a tendency toward attenuation. However, different results were obtained when using a more potent antagonist – AM251. This antagonist proves CB1 blockade's effectiveness in inhibiting cocaine's action on brain stimulation reward (250). Paradoxically, the non-selective cannabinoid agonist – WIN55, 212-2 – and the endocannabinoid transmission enhancer – AM404 – are also able to abolish cocaine's reinforcing effects as assessed by ICSS (252). Whether these apparently contradictory findings may indicate an inverse U-shape effect of CB1 stimulation function on rewarding properties of stimulants is not entirely clear. However, these results clearly indicate that cannabinoids might interfere with brain systems responsible for psychostimulants rewarding effects, and the mechanism underlying this phenomenon should be further explored.

Li and colleagues recently found significant reductions in DA levels in striatum of mice lacking the CNR1 gene, when compared to their wildtype counterparts following acute cocaine administration and during the basal state (253). This observation shows consistency with above-cited ICSS studies and with a previous report on the inhibition of cocaine-induced DA release in rats by CB1 antagonist SR141716A (254). In contrast, initial findings suggested that neither basal levels nor cocaine-induced increases in extracellular NAc DA of CB1 knockout mice differed from that of normal mice (255), and that CB1 inactivation by antagonists AM251 and SR141716A failed to alter the increase in extracellular NAc DA responsible for cocaine-mediated rewarding effects in rats (256, 257). Differences in experimental methods used to measure DA levels (voltammetry vs. in vivo microdialysis) and in the genetic background of the knockout animals (C57BL/6J vs. CD1) could account for such discrep-

ancies. Notably, compensatory neurobiological changes due to lack of CB1 receptors could explain the subnormal basal DA levels observed in Li et al. study. This subnormal basal DA levels could also have contributed to apparent attenuation of DA levels enhancement produced by cocaine. Overall, the extent to which ECBS interaction with psychostimulants-mediated reward effects depends on DA transmission seems limited, especially when CB1 is targeted. It remains a controversial issue, with subsequent reports of attenuation of cocaine-enhanced extracellular NAc DA activity by CB2 agonists JWH133 and GW405833 in mice (258), but not by the pharmacological FAAH inhibitor URB597 in rats (259) adding to the complexity of the matter (see Table 2).

The increase of DA neurotransmission in the NAc and other striatal regions responsible for psychostimulant-induced rewarding parallels the stimulation of locomotor activity following acute drug administration. Sensitization to hyperlocomotor responses produced by psychostimulants occurs after chronic treatment, reflecting adaptive changes in DA transmission and potentially correlating with drug-seeking and reinstatement behavior (254, 262). In various studies, neither genetic deletion (262–264) nor pharmacological inhibition of CB1 receptors by SR141716A (265, 266) altered cocaine's ability to induce acute motor effects or behavioral sensitization in rats and mice. However, a comparable number of reports described contradictory results, showing attenuation of both of these outcomes in CB1 knockout mice (253, 267) (see Table 3) as well as impairment of sensitization

Table 2 | Pharmacological inhibition of FAAH inhibition and properties of psychostimulants.

FAAH inhibitors	Drug	Animal	Outcome	Effects	Reference
AM404	Cocaine	Rat	Drug-induced lowering of brain reward/self-stimulation threshold	Impaired	Vlachou et al. (252)
			Drug-induced acute hyperlocomotion	Attenuated	Vlachou et al. (252)
URB597	Cocaine	Monkey	SA – effect of agonist after drug-taking extinction	No effect	Justinova et al. (260)
			SA – drug-taking behavior	No change	Justinova et al. (260)
		Rat	Cocaine-induced increase in VTA DA activity	Preserved	Luchicchi et al. (259)
			Cocaine-induced alterations in firing of NAc shell spiny neurons	Attenuated	Luchicchi et al. (259)
URB597, PMSF	Cocaine	Rat	SA – drug-seeking responses/intake	No change	Adamczyk et al. (261)
			SA – drug-induced reinstatement	Attenuated	Adamczyk et al. (261)
			SA – cue-induced reinstatement	Attenuated	Adamczyk et al. (261)

in animals pretreated with SR141716A (254, 268) or AM251 (267). Interestingly, although chronic cocaine use still induced sensitization in mice with invalidated CB1 receptors, sensitized response appeared somewhat changed when compared to control animals. Corbille et al. also found that AM251, unlike SR141716A (269), only impaired sensitization to cocaine after a single exposure, but not upon repeated administration. Similar experiments with cannabinoid agonists showed mixed results, as non-selective WIN 55,212-2 reduced cocaine's motor effects, probably in a non-CB1 mediated fashion (252, 270). Likewise, CB2 agonists JWH133 and GW405833 decreased both acute hyperlocomotion and sensitization in rats (258), which parallels findings observed in mice genetically overexpressing CB2 (271). Δ9-THC failed to alter cocaine's motor effects in rats (268, 272). Similarly, cannabinoid-amphetamine interactions studies demonstrate that acute cannabinoid exposure antagonizes amphetamine's locomotion effects in a dose-dependent manner in rats. On the other hand, chronic exposure to Δ9-THC induces sensitization to the psychomotor effects mediated by amphetamine in rats (273). Taken together, these experiments suggest that the acute motor stimulant

effects of psychostimulant and the induction of cocaine sensitization may not depend on endocannabinoid tone, even though CB1 receptors could play a minor modulating role in this regard [reviewed in Ref. (274)].

Overall, while some evidence of ECBS involvement in the neurobiological and behavioral correlates of psychostimulant reinforcing properties exists, influence of ECBS on acute psychostimulant reward is modest and probably involves a combination of mechanisms which may not directly involve DA activity in the NAc or CB1 receptors.

Influence of ECBS on acquisition and maintenance of psychostimulant-induced seeking behaviors

Models of conditioning such as the SA paradigm and the conditioned place-preference (CPP) procedure illustrate the reinforcing properties of drugs of abuse and demonstrate their ability to induce and maintain drug-seeking behaviors. Consistent with findings showing the limited involvement of the ECBS in the reinforcing properties of psychostimulants (see Influence of ECBS on Psychostimulants-Induced Behavioral and Reinforcing Effects), modulation of the ECBS appears to have a modest influence on acquisition and maintenance of psychostimulant-taking behavior in animals. In CPP experiments, while CB1 receptor deletion did not affect psychostimulant-induced place conditioning in mice, SR141716A impaired cocaine-, methamphetamine-, and MDMA-induced place conditioning in both rats and mice (262–265, 281, 286, 287). Difference in species, compensatory changes in the knockout animals due to the lack of CB1 receptors, as well as use of more intense conditioning and higher doses of drugs in the genetic deletion studies may have contributed to this discrepancy. This suggests that intensity of conditioning could overcome the effects of blocking ECBS signaling [reviewed in Ref. (274)].

It is important to note that the weaker cannabinoid antagonist CBD did not affect establishment of amphetamine-induced CPP in rats (290) and that the genetic overexpression of cannabinoid receptor CB2 impaired acquisition of both cocaine-induced CPP and SA (271). Thus the CPP model indicates that, although not directly interfering with the rewarding properties of psychostimulant drugs, ECBS could play a role in the perception and memory of psychostimulant reward, depending on environment-related factors.

In general, CB1 receptor invalidation does not seem to affect SA of psychostimulants. Experiments with genetic deletion of CB1 show conflicting results, as both knockout and SR141716A-treated mice still acquired amphetamine- and cocaine-taking behavior in restrained mobility conditions (266, 275), whereas knockout mice showed impaired SA behavior in other protocols (255, 258). Overall, results suggest that learning SA behavior might not require extensive ECBS involvement. Maintenance of such behavior may not depend on CB1 either, as drug-taking responses under a fixed-ratio schedule in animals that had already acquired cocaine SA remained unaffected after CB1 blockade by SR141716A in mice (266, 283), monkeys (280), and rats (250, 256, 269, 283) and by AM251 in rats (250, 277). Only one contradicting report exists in which AM251 decreased methamphetamine SA in conditioned rats (278). Cannabinoid signaling enhancement by the pharmacological FAAH inhibitors – URB597 and PMSF – also failed to affect maintenance of fixed-ratio drug-taking behavior in rats (261) and monkeys (260). On the other hand, cannabinoid stimulation by CB1 agonists had significant effects in several studies.

Table 3 | Effects of CB1 cannabinoid receptor deletion and properties of psychostimulants; CB1 receptor antagonists and properties of psychostimulants.

Genotype	Drug	Animal	Outcome	Effects	Reference
CB1 KO	Cocaine	Mouse	Drug-induced acute hyperlocomotion	Preserved	Martin et al. (262), Houchi et al. (263), Miller et al. (264)
			Drug-induced acute hyperlocomotion	Attenuated	Corbille et al. (267), Li et al. (253)
			Drug-induced motor sensitization	Preserved	Martin et al. (262)
			Drug-induced motor sensitization	Attenuated	Corbille et al. (267)
			Drug-induced increase in NAc DA levels	Preserved	Soria et al. (255)
			Drug-induced increase in NAc DA levels	Attenuated	Li et al. (253)
			CPP – behavior acquisition under chronic unpredictable stress exposure	Preserved	Martin et al. (262), Houchi et al. (263), Miller et al. (264)
				Enhanced	Miller et al. (264)
			SA – behavior acquisition in restrained mobility protocol	Impaired	Soria et al. (255), Xi et al. (258)
				Preserved	Cossu et al. (275)
			SA – breaking point under PR schedule	Decreased	Soria et al. (255)
	Amphet.		Drug-induced acute hyperlocomotion	Preserved	Houchi et al. (263)
			Drug-induced acute hyperlocomotion	Attenuated	Corbille et al. (267)
			Drug-induced motor sensitization	Attenuated	Corbille et al. (267)
			SA – behavior acquisition in restrained mobility protocol		
				Preserved	Cossu et al. (275)

Antagonist	Drug	Animal	Outcome	Effects	Reference
AM251	Cocaine	Mouse	Drug-induced acute hyperlocomotion	Attenuated	Corbille et al. (267)
			Drug-induced motor sensitization (induction)	Attenuated	Corbille et al. (267)
			Drug-induced motor sensitization (expression)	Preserved	Corbille et al. (267)
			CPP – drug-induced reinstatement	Preserved	Vaughn et al. (276)
			CPP – stress-induced reinstatement	Impaired	Vaughn et al. (276)
		Rat	Drug-induced lowering of brain reward/self-stimulation threshold	Attenuated	Xi et al. (250)
			Drug-induced increase in NAc DA levels	Preserved	Xi et al. (257)
			Drug-induced increase in NAc glutamate	Attenuated	Xi et al. (257)
			SA – drug-induced reinstatement	Attenuated	Xi et al. (257), Adamczyk et al. (277)
			SA – cue-induced reinstatement	Attenuated	Adamczyk et al. (277)
			SA – drug-seeking responses/intake	No change	Xi et al. (250), Adamczyk et al. (277)
			SA – breaking point under PR schedule	Decreased	Xi et al. (250)
	METH	Rat	SA – drug-seeking responses/intake	Decreased	Vinklerova et al. (278)
SR141716A	Amphet., cocaine	Gerbils	Drug-induced acute hyperlocomotion	Decreased	Poncelet et al. (279)
			Reinstatement of drug-seeking	Decreased	Poncelet et al. (279)
	Cocaine	Monkey	SA – drug-seeking responses/intake	No change	Tanda et al. (280)
	Mouse		Drug-induced acute hyperlocomotion	Attenuated	Gerdeman et al. (268)
				Preserved	Lesscher et al. (266)
			Drug-induced motor sensitization (induction)	Attenuated	Gerdeman et al. (268)
				Preserved	Lesscher et al. (266)

(Continued)

Table 3 | Continued

Antagonist	Drug	Animal	Outcome	Effects	Reference
			Drug-induced motor sensitization (expression)	Preserved	Gerdeman et al. (268)
			Drug-induced motor sensitization (maintenance – specific to a drug-paired environment)	Reversed	Gerdeman et al. (268)

CPP – behavior acquisition Impaired Yu et al. (281)
CPP – drug-induced reinstatement Impaired Yu et al. (281)
SA – behavior acquisition Preserved Lesscher et al. (266)
SA – "extinction burst responding" Attenuated Ward et al. (282)
SA – time for behavior extinction Decreased Ward et al. (282)
SA – cue-induced reinstatement Attenuated Ward et al. (282)
SA – drug-seeking responses/intake No change De Vries et al. (283), Lesscher et al. (266)
SA – breaking point under PR schedule Decreased Soria et al. (255)
Rat Drug-induced acute hyperlocomotion Preserved Chaperon et al. (265)
Attenuated Cheer et al. (254)
Drug-induced motor sensitization (expression) Attenuated Filip et al. (269)
Drug-induced lowering of brain reward/self-stimulation threshold
Preserved Vlachou et al. (251), Xi et al. (250)
Drug-induced decrease in VP GABA efflux Preserved Caille and Parsons (256)
Drug-induced increase in NAc DA levels Preserved Caille and Parsons (256)
Suppressed Cheer et al. (254)
Drug discrimination Preserved Filip et al. (269)
SA – drug-seeking responses/intake No change De Vries et al. (283), Caille and Parsons (256), Filip et al. (269), Xi et al. (250)
SA – breaking point under PR schedule No change Xi et al. (250)
Decreased Orio et al. (284)
SA – drug-induced reinstatement Attenuated De Vries et al. (283), Filip et al. (269)
SA – HU210-induced reinstatement Attenuated De Vries et al. (283)
SA – cue-induced reinstatement Attenuated De Vries et al. (283), Filip et al. (269)
SA – stress-induced reinstatement Preserved De Vries et al. (283)
CPP – behavior acquisition Impaired Chaperon et al. (265)
CPP – behavior expression Preserved Chaperon et al. (265)
MDMA Mouse CPP – drug-induced reinstatement Increased Daza-Losada et al. (285)
CPP – behavior acquisition Impaired Rodriguez-Arias et al. (286)
Rat CPP – behavior acquisition Impaired Braida et al. (287)
SA – drug-seeking responses/intake Increased Braida and Sala (288)
METH Mouse CPP – behavior acquisition Impaired Yu et al. (281)
CPP – drug-induced reinstatement Impaired Yu et al. (281)
Rat Drug-induced reinstatement of drug-seeking behavior
Attenuated Anggadiredja et al. (289)
Cue-induced reinstatement of drug-seeking behavior
Attenuated Anggadiredja et al. (289)

Indeed, WIN 55,212-2 increased acquisition of MDMA SA in mice (286) and exposure to CP55,940 enhances development of cocaine SA in female rats (291). THC failed to alter acquisition of cocaine SA and amphetamine SA in monkeys (272) and rats (290), respectively. In regard to maintenance of drug intake in animals with SA behavior, cannabinoid agonists decreased drug-taking responses in rats – CP55,940 diminished MDMA intake (288) and WIN55,212-2 decreased cocaine administration (292) – and in monkeys – Δ9-THC also decreased cocaine intake (272). Fattore et al. first interpreted the shift in psychostimulant intake produced by CB1 agonists as indicative of a synergistic action of CB1 stimulation on reinforcing properties of the drugs, which, incidentally, could account for the frequent use of cannabis among human psychostimulants users (292). Complementary experiments using progressive-ratio schedules also reveal interaction of CB1 receptors with psychostimulant-induced reinforcing properties. In PR schedules, both genetic deletion and antagonist

treatment of CB1 receptors produce a decrease in the maximal effort mice provided to self-administer cocaine, as made apparent by decreases in breaking point measures induced by SR141716A (255, 277, 284) and by AM251 (250) (SR141716A producing no effect in this specific report). This adds to the evidence that the CB1 receptors, while not indispensable for acquisition or maintenance of cocaine-seeking behavior, may exert a specific modulation on motivation and reward salience in psychostimulant addiction.

Role of ECBS in extinction and reinstatement of drug-taking behaviors

Although the mechanism used by endocannabinoid signaling to modulate psychostimulant reward and acquisition or maintenance is still the subject of debate, some form of consensus exists in the literature about the pivotal role of the ECBS in extinction and reinstatement in animal behavioral models of psychostimulant addiction [reviewed in Refs. (89) and (274)]. In conditioning procedures, extinction refers to the learning phase that follows the removal of the reinforcer (i.e., psychostimulant drugs), during which rates of conditioned responses (i.e., SA or CPP) progressively decline back to pre-conditioning levels. After drug-seeking behavior becomes extinct, several behavioral phenomena can reinstate drug-seeking behavior. These include not only re-exposure to the abused drug itself, but also exposure to contextual cues associated with previous drug administration and to environmental stressors (274, 293).

In a recent study from Ward et al. mice treated with SR141716 after removal of cocaine in a SA paradigm altered the burst in cocaine-seeking observed in the initial phase of extinction learning, while decreasing the time required to achieve complete extinction of cocaine-seeking behavior when compared to vehicle-treated mice (282). CB1 blockade by SR141716A also significantly decreases cue-induced reinstatement of cocaine SA behavior following extinction, supporting similar reports of attenuation of cue-induced reinstatement of cocaine-seeking by CB1 antagonism in rats (269, 277, 283). Evaluation of reinstatement of SA behavior induced by drug-priming produced similar results: SR141716A blocks cocaine-induced reinstatement (269,283),and both AM251 (257, 277) and SR141716A inhibit methamphetamine-induced reinstatement (289). It is worth noting that CB2 antagonism also has an anti-reinstatement effect in cocaine-primed, but not in cue-exposed rats (277). In CPP models, CB1 blockade by SR141716A, but not by AM251 (276), impaired drug-induced reinstatement in cocaine-conditioned mice (281). SR141716A also impaired methamphetamine-induced reinstatement of CPP (281). Few studies focused on stress-induced reinstatement of psychostimulant-seeking.Vaughn et al. recently found that AM251 reverses stress-induced CPP (276). De Vries et al. could not find an impact of SR141716A on stress-induced SA reinstatement (283).

Stimulation of CB1 receptors produced opposite results to those obtained in pharmacological blockade experiments (see Table 4). WIN55,212-2 increases time for extinction of CPP and enhances drug-induced reinstatement in MDMA-conditioned mice (285, 286). Similarly, Δ9-THC increases cue-induced reinstatement of methamphetamine SA in rats (289). However, studies from Parker et al. and Adamczyk et al. complexify the interpretation of these results (290). Indeed, Adamczyk's group showed that FAAH inhibition impairs cue- and drug-induced reinstatement of cocaine SA. Using the place-preference conditioning paradigm,

Parker et al. have assessed the potential of both exogenous cannabinoids – Δ9-THC and CBD – to potentiate the extinction of cocaine- and amphetamine-induced CPP (290). After the establishment of cocaine-induced and amphetamine-induced place preference, rats were given an extinction trial, 30 min prior to which they were injected with a low dose of Δ9-THC, CBD, or vehicle. During conditioning trials, researchers also injected rats with cannabinoids, or vehicle, prior to an amphetamine injection, to determine the effects of Δ9-THC or CBD on the establishment and expression of a place preference. Results indicate that Δ9-THC and CBD potentiate the extinction of stimulant-CPP learning, which is not mediated by an alteration of learning or retrieval. CBD does not have a reinforcing or hedonic property on its own, suggesting that it does not have the addictive potential of Δ9-THC, a significant advantage in terms of therapeutic use. The non-reinforcing aspect of CBD has been replicated in studies looking at the co-administration of CBD and Δ9-THC (212, 294). These discrepancies probably result from the lack of receptor selectivity in the methods used to enhanced cannabinoid signaling. Nonetheless, these experiments support a significant involvement of ECBS in the extinction and reinstatement of behaviors related to psychostimulant addiction. Overall, the positive results of CB1 antagonists on extinction and prevention of reinstatement of psychostimulant SA, combined with their lack of reinforcing properties, suggest a therapeutic potential for CB1 modulation in treatment of psychostimulant addiction.

CONCLUSION

A growing number of studies have investigated the neurobiological and behavioral mechanisms leading to psychostimulants dependence. A key feature of drug dependence is the relapse to drug use even after long period of abstinence. While greatly improved in recent years, treatment strategies for psychostimulants have

Table 4 | CB1 receptor agonists and properties of psychostimulants.

Agonists	Drugs	Models	Outcome	Effects	Reference
CP55,940	Cocaine	Mouse SA – effect of agonist after drug-taking extinction	No effect		Vaughn et al. (276)
		Rat SA – behavior acquisition following exposure during adolescence in female specimen	Enhanced		Higuera-Matas et al. (291)
	MDMA	Rat SA – drug-seeking responses/intake	Decreased		Braida and Sala (288)
HU210	Cocaine	Rat SA – effect of agonist after drug-taking extinction	Reinstatement		De Vries et al. (283)
Δ9-THC	Amphet.	Rat CPP – behavior acquisition	Preserved		Parker et al. (290)
		CPP – behavior extinction	Potentiated		Parker et al. (290)
	Cocaine	Monkey SA – effect of agonist after drug-taking extinction	Reinstatement		Justinova et al. (260)
		Mouse Drug-induced motor sensitization	Preserved		Gerdeman et al. (268)
		Rat Drug-induced acute hyperlocomotion	Preserved		Panlilio et al. (272)
		Drug-induced motor sensitization	Preserved		Panlilio et al. (272)
		Drug-induced anxiety	Increased		Panlilio et al. (272)
		SA – behavior acquisition	Preserved		Panlilio et al. (272)
		SA – drug-seeking responses under PR schedule	Decreased		Panlilio et al. (272)
		CPP – behavior extinction	Potentiated		Parker et al. (290)
	METH	Rat SA – cue-induced reinstatement	Increased		Anggadiredja et al. (289)
		SA – drug-induced reinstatement	Attenuated		Anggadiredja et al. (289)
WIN-55	Cocaine	Rat Drug-induced acute hyperlocomotion	Attenuated		Przegalinski et al. (270), Vlachou et al. (252)
		Drug-induced lowering of brain reward/self-stimulation threshold	Impaired		Vlachou et al. (251)

SA – drug-seeking responses/intake Decreased De Vries et al. (283)
MDMA Mouse CPP – behavior acquisition Increased Rodriguez-Arias et al. (286)
CPP – time for behavior extinction Increased Rodriguez-Arias et al. (286)
CPP – drug-induced reinstatement Increased Rodriguez-Arias et al. (286), Daza-Losada et al. (285)

yet to address effectively drug-seeking behaviors linked to high rates of relapse, persistent drug use as well as subsequent health, mental, and social problems. There is consequently an urgent need for researchers to identify compounds that might help patients (1) initiate abstinence and (2) avoid relapse. The ECBS appears to play a critical role in dependence to psychostimulants and experimental studies are now providing evidence that while it does not participate in the primary reinforcing properties of psychostimulants, it reliably modulates relapse to drugs. Interestingly, emerging human data supports a role for ECBS modulation in vulnerability to psychostimulant addiction, and more significantly in addictive behaviors among dependent individuals. Accumulating evidence thus points to the ECBS as a critical target for the development of pharmacotherapies for the treatment of addiction to psychostimulants. Given the various neuropharmacological actions of exogenous cannabinoids, and their ability to modulate the acute reinforcing effects of drugs, data on Δ9-THC and CBD is particularly promising as to the potential use of cannabinoids in relapse prevention strategies for psychostimulant-dependent individuals. The effects of these compounds on stimulant use outcomes in humans remains to be clearly established and could be assessed with well-designed controlled trials. The neurobiological correlates of cannabinoids' impact on stimulant-seeking behaviors could also be examined with neuroimaging studies in stimulants dependent individuals. Among potential barriers, social and scientific acceptability of cannabinoid-based therapy, side effects profiles, as well as addictive potential of certain cannabinoid such as Δ9-THC, have to be taken into consideration.

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